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Can polyphenols' metabolites ameliorate the outcome of Diabetic Retinopathy?

Dissertação para obtenção do Grau de Mestre em Biotecnologia

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Dissertação apresentada para a obtenção do Grau de Mestre em Biotecnologia, pela Universidade Nova de Lisboa, Faculdade de Ciências e Tecnologia

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Can polyphenols' metabolites ameliorate de outcome of DR?

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Resumo

A prevalência de Diabetes *Mellitus* (DM) na população mundial tem vindo a aumentar e a Retinopatia Diabética (RD), uma doença associada à disrupção da barreira hemato-retiniana, é a complicação ocular mais comum relacionada com esta doença em países desenvolvidos. Uma exposição crónica a hiperglicemia e hipóxia é crucial para a fisiopatologia da RD, que em estágios avançados provoca neovascularização da retina e consequentemente, perda de visão. Não há cura para a RD, existem apenas tratamentos invasivos como fotocoagulação a laser e terapias anti-VEGF, que apresentam inúmeras desvantagens e apenas atrasam a progressão da doença. Vários estudos relacionados com esta doença descrevem um desequilíbrio entre fatores pro- e anti-angiogénicos, o que promove o processo de angiogénese patológica, característico da RD. Foi também descrito que pacientes com RD apresentam alterações fisiológicas consistentes com um estado de inflamação crónica. Desta forma, e sabendo que a angiogénese patológica e a inflamação crónica são das principais características da RD, desenvolver tratamentos com base nestes processos poderá constituir uma alternativa terapêutica.

Os compostos polifenólicos têm sido utilizados em vários paradigmas da doença devido ao seu potencial efeito terapêutico. Existem estudos que demonstram que o sulfato de catecol (Cat-sulf) e o sulfato de pirogalol (Pyr-sulf) reduzem a expressão de marcadores de inflamação e que são capazes de atravessar a barreira hematoencefálica em modelos *in vitro.*

Desta forma, o objetivo deste estudo é avaliar o efeito do Cat-sulf e do Pyr-sulf na expressão de marcadores pro- e anti-angiogénicos, na expressão do transportador de glucose na retina (GLUT1) e em marcadores de inflamação, quer *in vitro,* utilizando uma linha celular da do epitélio pigmentar da retina, quer *in vivo*, usando um modelo de ratinho para RD. Os nossos resultados mostram que nas concentrações testadas, o Cat-sulf influenciou negativamente a viabilidade das células e foi, assim, excluído do restante estudo. Para o Pyr-sulf os resultados mostraram um efeito positivo sobre a viabilidade celular e ainda a capacidade de aumentar os níveis de mRNA de PEDF e diminuir a expressão da proteína VEGF sob condições diabéticas. Em condições diabéticas, a expressão do GLUT1 é aumentada e o tratamento com Pyr-sulf, embora sem significado estatístico, diminui a expressão de GLUT1 em condições diabéticas *in vitro*, destacando o efeito benéfico deste metabolito fenólico no controlo do transporte de glucose. Verifica-se também que em células do epitélio pigmentar da retina tratadas com Pyr-sulf há uma diminuição de marcadores de inflamação, confirmando o potential anti-inflamatório deste composto. Para complementar estes resultados *in vitro,* mostramos que o metabolito diminui a expressão da proteína pro-inflamatória Iba1 em ratinhos diabéticos com RD.

No seu conjunto, estes resultados evidenciam o potencial do Pyr-sulf para tratamento de doenças associadas com inflamação crónica e angiogénese patológica, como a DR.

Palavras chave: Diabetes Mellitus; Retinopatia Diabética; Angiogénese; Inflamação; metabolitos fenólicos.

Abstract

The prevalence of *Diabetes Mellitus* worldwide continues to increase and Diabetic Retinopathy (DR), a blood-retinal barrier disorder, is its most common ocular complication in developed countries. Chronic exposure to hyperglycemia and hypoxia is essential to the pathophysiology of DR, that in advanced stages leads to neovascularization and consequently vision loss. There is no cure for DR, only symptomatic care with invasive treatments, such as laser photocoagulation, which have several drawbacks, and anti-VEGF (vascular endothelial growth factor) therapies, which do not halt the progression of the disease.

Several studies report an imbalance between pro-angiogenic and anti-angiogenic factors, which promotes the pathological angiogenesis characteristic of DR. It was also reported that patients with DR have physiological changes consistent with chronic inflammation. Since abnormal angiogenesis and chronic inflammation are considered important hallmarks of DR, it is critical to develop efficient therapies.

Phenolic metabolites have been used in several disease paradigms due to its potential therapeutic effect. It is reported that Cathecol-O-sulfate (Cat-sulf) and Pyrogallol-O-sulfate (Pyr-sulf) were shown to reduce inflammation markers and cross blood brain barrier in *in vitro* models.

Therefore, the aim of this study is to evaluate the effect of Cat-sulf and Pyr-sulf in the expression of pro- and anti-angiogenic markers, in the expression of retina glucose transporter (GLUT1) and in inflammation biomarkers. *In vitro*, using a retinal cell line, and *in vivo*, using a mouse model of DR.

Our results show that in the tested concentrations, Cat-sulf had a negative influence on the viability of D407 RPE cells and was excluded for the rest of this study. For Pyr-sulf our results have shown a positive effect on cell viability and it increases the mRNA levels of the PEDF (Pigment Epithelium–Derived Factor) and decreases VEGF protein expression under diabetic conditions.

It is well known that in diabetic conditions the expression of the GLUT1 is increased. Treatment with Pyr-sulf, although not statistically significant, decreases GLUT1 expression under diabetic conditions in D407 RPE cells, highlighting the beneficial effect of this phenolic metabolite in controlling glucose transport.

Furthermore, in RPE cells treated with Pyr-sulf the expression of inflammatory markers under hypoxic conditions decreases, confirming the anti-inflammatory effect of this phenolic metabolite. To support our *in vitro* studies, we have shown that in $Ins2^{Akita}$ mice, a model for type I diabetes and DR, Pyr-sulf decreases the expression of the pro-inflammatory protein Iba1.

Taken together, these results shown the great potential of Pyr-sulf as treatment for diseases associated with chronic inflammation and abnormal angiogenesis, such as DR.

Keywords: Diabetes Mellitus; Diabetic Retinopathy; angiogenesis; inflammation; phenolic metabolites.

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List of Abbreviations

BSA - Bovine Albumin Serum

Cat-sulf - Cathecol-*O*-sulfate

cDNA - Complementar Deoxyribonucleic Acid

DFO - Desferrioxamine

DM - Diabetes *Mellitus*

DMEM - Dulbecco's Modified Eagle's Medium

DNA - Deoxyribonucleic Acid

DR - Diabetic Retinopathy

FBS - Fetal Bovine Serum

GFAP - Glial Fibrillary Acidic Protein

GLUT1 - Glucose Transporter 1

HCl - Hydrochlorid acid

IL-1β – Interleukine-1β

IL-8 – Interleukine-8

Iba1 – Ionized Calcium-Binding Adapter molecule 1

Ins2 – Insulin II gene

kDa - kilodaltons

MTT - 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)

mRNA - Messenger RNA

PBS - Phosphate-Buffered Saline

PCR - Polymerase Chain Reaction

PEDF - Pigment Epithelium–Derived Factor

Pyr-sulf – Pyrogallol-*O*-sulfate

qRT-PCR - Quantitative Real Time PCR

RNA - Ribonucleic Acid

ROS - Reactive Oxygen Species

RPE – Retinal Pigment Epithelium

rpm - rotation per minute

TBS-T - Tris-Buffered Saline, 0.1% Tween 20

TNFα - Tumor Necrosis Factor alpha

VEGF - Vascular Endothelial Growth Factor

VEGFR - Vascular Endothelial Growth Factor Receptor

WT - Wild Type

1. Introduction

1.1. Diabetes

Nowadays, the sedentary life, obesity, unhealthy food, smoking, and alcohol consumption lead to increased prevalence of diseases such as Diabetes *Mellitus* (DM) (Hu, 2011). DM is a group of metabolic disorders mainly characterized by high levels of glucose in blood resulting from defects in insulin secretion and/or action. DM is divided in two categories: type 1 diabetes, characterized by autoimmune destruction of pancreatic insulin-producing β-cells and consequently absolute deficiency in insulin secretion; type 2 diabetes, much more prevalent, combines the resistance to insulin action with abnormal secretion. Both, type 1 and type 2 diabetes are related with abnormal glucose homeostasis; however there are other types of diabetes, such gestational diabetes, characterized by glucose intolerance during pregnancy and diabetes caused by genetic defects (American Diabetes, 2010).

Common to all types of diabetes is the chronic hyperglycemia further associated with dysfunction and damage of different organs, in which the eye is included.

1.2. The eye

The eye is the organ responsible for vision, which is composed by several structures involved in the vision cycle. The retina is an inner layer of the posterior cavity of the eye that receives the light signal, converts it to chemical signals and sends these to the brain through the optic nerve. In greater detail the retina is divided in ten different layers composed by three major cell types: photoreceptors, several types of neural cells, and retinal pigment epithelium (RPE) cells (Figure 1.1).

Photoreceptors cells are light sensitive neurons in the retina, responsible for transducing the light stimulus in an electric signal. There are two distinct subtypes of photoreceptors: rods and cones. The rods photoreceptors are very sensitive to low-light and cones operate with bright light and are responsible for color perception and central vision. Neural cells (ganglion, horizontal, bipolar and amacrine cells) are involved in the transduction of the eletrical signal to the optic nerve and then to the brain. The bipolar and horizontal cells receive the signal from the photoreceptors, transfer to the amacrine cells which interact with the ganglion cells, the output neurons (Naik, Mukhopadhyay and Ganguli, 2009; Veleri *et al.*, 2015). Its axons transmit the information from the retina to the brain.

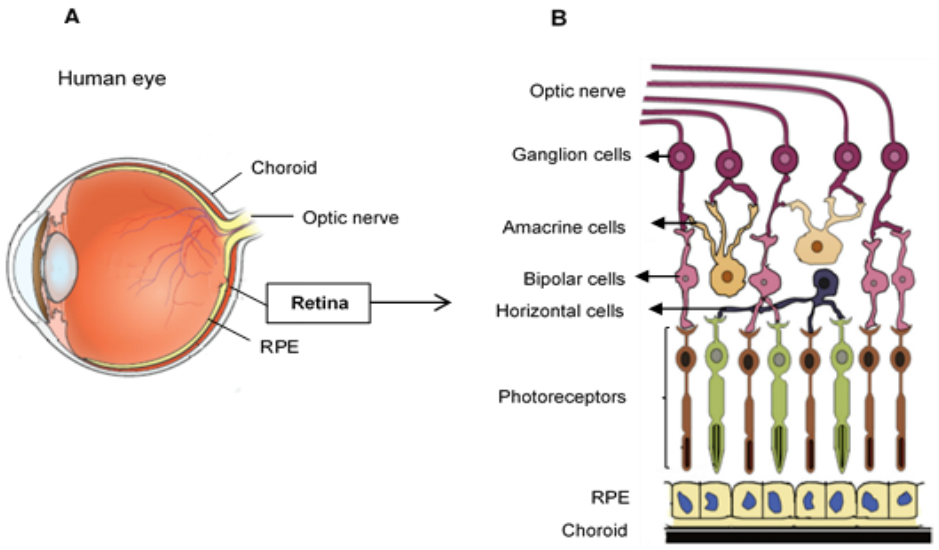

Figure 1.1 Structure of the eye and different cells of the retina. (A) schematic representation of human eye and its major structures. (B) Retinal cells organization composed by choroid, RPE, photoreceptors, different neural cells (horizontal, bipolar, amacrine and ganglion cells) and the optic nerve. Adapted from (Naik, Mukhopadhyay and Ganguli, 2009; Veleri *et al.*, 2015).

The RPE is formed by pigmented cuboidal cells that makes part of the retina/blood barrier surrounded by the choroid. These cells have the capacity of absorb scattered light and serves as a barrier between the choroid and the photoreceptors. RPE is also responsible for the transport of glucose and other nutrients to the photoreceptors, which makes RPE crucial in its maintenance (Veleri *et al.*, 2015). Besides that, RPE secretes growth and neurotrophic factors responsible for maintaining the structural integrity of the retina which is essential for visual function (reviewed in Araújo, Santos and Silva, 2018; Strauss, 2005). One of the most important molecules secreted by the RPE is the pigment epithelial-derived factor (PEDF), a potent anti-inflammatory and anti-angiogenic protein (reviewed in Araújo, Santos and Silva, 2018), crucial in physiology and pathophysiology of RPE. The main functions of RPE are resumed in Figure 1.2.

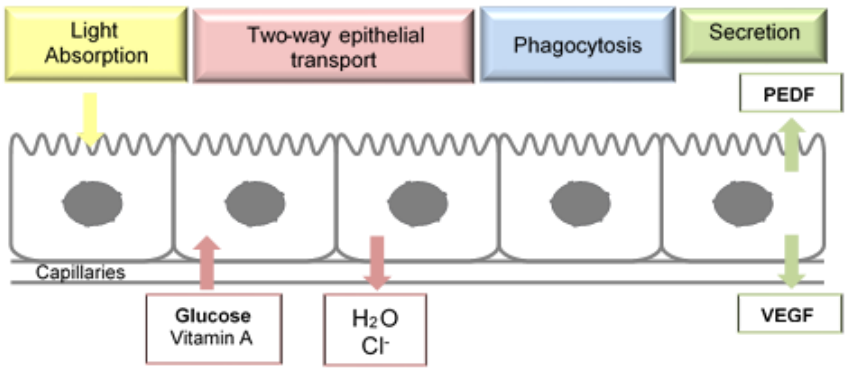

Figure 1.2 Retinal Pigment Epithelium (RPE) main functions: scattered light absortion, two-way epithelial transport, phagocytosis and secretion of growth neurotrophic factors such as VEGF and PEDF. Adapted from (Strauss, 2005).

Diseases affecting the retina are blinding disorders influenced by genetic and/or environmental factors altogether contributing to more than 25% of blindness cases (Resnikoff *et al.*, 2004). Acquired retinal disorders include Age-related Macular Degeneration, *Retinitis Pigmentosa*, Glaucoma and Diabetic Retinopathy (DR) that results from a combination of aging, environmental and genetic factors that damage the retina and RPE. Since photoceptors and RPE cells are affected in these diseases, they can be suitable targets for therapies.

The anatomical location of the eye makes it an excellent therapy target because it allows easy acessibility and due to its small size the amount of therapeutic required is reduced (Bainbridge, Tan and Ali, 2006; Borrás, 2003).

1.3. Diabetic Retinopathy

The prevalence of DM worldwide continues to increase and DR is the most common ocular complication of this disease in developed countries (Fong *et al.*, 2004). DR is a chronic and progressive disease characterized by ischemia, microaneurysms, hemorrhages, neovascularization and increased vascular permeability. This disease leads to vision loss and affects mostly working-age adults, because it develops several years after the onset of diabetes (Cheung, Mitchell and Wong, 2010).

Clinically, there are two types of DR: non-proliferative, associated with early stages and proliferative, related with advanced stages. In the non-proliferative form there is an abnormal blood flow, promoting changes in the retina vascular permeability. In advanced stages ischemia can develop, leading to proliferative DR. The proliferative form is related with hypoxia and neo-angiogenesis, which leads to formation of new, abnormal blood vessels in the eye, called neovascularization. The new blood vessels are usually weak, fragile and leaky which allows proteins, fluids and debris to enter the retina (Cheung, Mitchell and Wong, 2010; Fong *et al.*, 2004). This type of retinopathy is usually more severe, because hemorrhages in the retina lead to severe vision loss.

At present, there is no efficient treatment for DR. Glycemic control helps to slow the progression and, in advanced stages, invasive therapies like photocoagulation and ocular injections of anti-angiogenic agents are applied with several negative effects such as peripheral vision loss and damage of neural tissues (Caldwell *et al.*, 2003).

The pathophysiological mechanism behind DR is not yet fully known but is believed that the chronic exposure to hyperglycemia and hypoxia initiates a cascade of biochemical and physiological changes that lead to neovascularization and retina dysfunction (Cheung, Mitchell and Wong, 2010). Most of the studies found in the literature are focused on the effects of DR in RPE cells, because these cells are crucial to the homeostasis of the neuroretina and to transport important nutrients.

1.3.1. Pathophysiology of DR

Several mechanisms have been proposed to modulate the pathogenesis of this disease, like the modulation of angiogenesis and inflammation.

1.3.1.1. Angiogenesis

Angiogenesis is the formation of new blood vessels from pre-existing ones, requiring endothelial cell migration, growth and differentiation, that begins in the embryonic stage and continues until death, being active in both physiological and pathological situations (Risau, 1997). All tissues in the body are formed of blood vessels, responsible for exchange of nutrients and metabolites, and clean waste products. The oxygen levels have a pivotal role in the regulation of angiogenesis. In sprouting angiogenesis, the oxygen sensing mechanisms detect a certain level of hypoxia (oxygen privation) and stimulate the production of a pro-angiogenic factor, the vascular endothelium growth factor (VEGF), a signaling molecule that binds to a specific receptor in endothelial cells membrane and promotes the formation of the new blood vessels to satisfy tissues metabolic requirements (Nagy *et al.*, 2008). In pathological angiogenesis, VEGF is overexpressed (Fong *et al.*, 2004) leading to an abnormal and uncontrolled formation of new blood vessels which can cause irreversible damage, such as neovascularization in the eye and consequently vision loss (Cheung, Mitchell and Wong, 2010).

1.3.1.1.1. VEGF

VEGF or VEGF-A (42 kDa), is a protein from a large family of angiogenic proteins, that is secreted by different cells in retina, mostly by the RPE. The main functions of these proteins are the regulation of angiogenesis and vascular permeability. The VEGF signal involves the binding of this factor to specific tyrosine-kinase receptors called VEGFR (Figure 1.3), promoting proliferation and migration of endothelial cells, and the formation of new blood vessels (Nagy *et al.*, 2008). VEGF is also involved in the inflammatory response since is strongly expressed in wound healing, where microvascular permeability and angiogenesis are increased (Ferrara, Gerber and Lecouter, 2003). In pathologies associated with abnormal angiogenesis, like DR, standard treatments associated with the inhibition of VEGF have been successfully used to slow disease progression and to reduce the risk of vision loss (Ni and Hui, 2009). However these therapies are limited because the injections must be repeated frequently and has complications associated, such as RPE and photoreceptors degeneration and increased ocular pressure (Araújo, Santos and Silva, 2018; Ni and Hui, 2009; Simão *et al.*, 2016).

In a healthy eye, the levels of pro-angiogenic and anti-angiogenic factors are balanced and promote the control of new blood vessels formation. In addition to VEGF overexpression, there is a downregulation of anti-angiogenic factors, such as PEDF.

1.3.1.1.2. PEDF

PEDF (50 kDa) is a protein from the serine protease inhibitor (serpin) super family. During evolution PEDF lost the protease inhibitory activity and gained others properties like anti-oxidative, neuroprotective, anti-inflammatory and anti-angiogenic (Araújo, Santos and Silva, 2018; Becerra and Notario, 2013; Dawson, 1999). PEDF is a potent anti-angiogenic factor through apoptotic effects on endothelial cells and, in the eye, overexpression of PEDF prevents the formation of new blood vessels, conferring protection to the retina. The molecular mechanisms involved in PEDF functions are based on its interactions with cell-surface receptors (Becerra and Notario, 2013). Related with its properties, the therapeutic potential of PEDF was considered.

The imbalance between these pro-angiogenic and anti-angiogenic proteins in DR is considered responsible for the vascular alterations and consequently vision loss (Farjo and Ma, 2010). The signaling events behind PEDF activity in cells are represented in Figure 1.3.

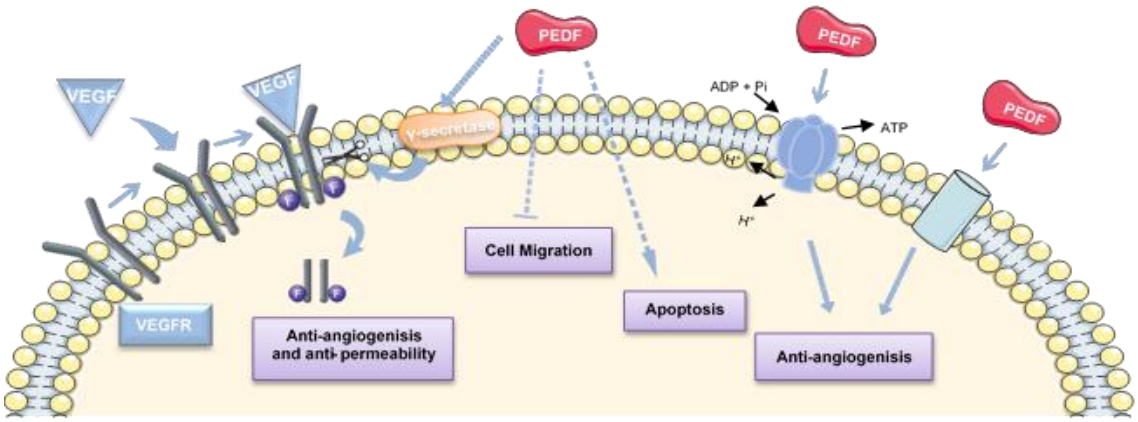

Figure 1.3 Signalling events of PEDF in cells. VEGF binds to the specific receptor, VEGFR, which becomes phosphorylated and activated. PEDF increases the levels of γ-secretase which promotes the cleavage of VEGFR and inhibit the VEGF-driven angiogenesis and permeability. PEDF can also activate a specific pathway to inhibit endothelial cell migration and induces apoptosis. PEDF is a ligand to two proteins on endothelial cells, that results in angiogenic responses. Adapted from (Becerra and Notario, 2013).

1.3.1.2. Inflammation

Inflammation is a biological response to harmful stimuli that protects cells of permanent damage, but chronic inflammation can be harmful (Ibrahim *et al.*, 2011). This natural process allows the immune system to recognize a pathogen by its specific binding to pattern recognition receptors. The activation of these receptors results in the production or recruitment of specific cytokines, which induces the expression of other pro-inflammatory proteins that are controlled at gene transcription level. This increased expression of pro-inflammatory proteins is regulated trough the activation of pro-inflammatory transcription factors (Tang and Kern, 2011).

Several physiologic and molecular changes that are consistent with inflammation have been found in the retinas of diabetic animals and patients (Brucklacher *et al.*, 2008), which has been widely reviewed in (Adamis and Berman, 2008; Kaul *et al.*, 2010; Kern, 2007). Understanding the mechanisms linking inflammation to DM and related complications is very important to develop new strategies to prevent them, by targeting inflammatory pathways.

Inflammation contributes to DM by causing insulin resistance and it is intensified in high concentrations of glucose. This happens because several cytokines activated in inflammatory processes have the capacity of develop insulin resistance (Tanaka, Narazaki and Kishimoto, 2014). Hyperglycemia itself has been regarded as pro-inflammatory environment, so chronic inflammation may play a critical role in the development of early stages of DR (Tang and Kern, 2011).

Since RPE has an important role in the maintenance of visual function, acknowledgment of the inflammatory process occurring in these cells could lead to new therapeutic targets. Retinal cells have characteristics that allow them to mediate the immune response in the eye, such as described above (Tang and Kern, 2011). However, the understanding of the inflammatory process behind DR pathogenesis is poor and needs to be expanded. Several key players have been indicated as part of the inflammatory process of DR, as follows.

1.3.1.2.1. Microglia cells

Retinal microglia or Müller cells are the most important glial cell type in the retina because they act like macrophages, forming the first active immune defense in the neural retina. Microglia activation results from very sensitive calcium transport channels to small alterations in extracellular calcium. This activation promotes a cytokine-induced activation cascade to help microglia in inflammation process. If microglia remain active, the cytokines damaged other cell types, which in this context can lead to retinal degeneration and chronic inflammation.

During the activation of microglia, some specific proteins, like the ionized calcium-binding adapter molecule 1 (Iba1, 17 kDa) are up-regulated, promoting the migration and proliferation of cells involved in the vascular process and the deregulation of the cell cycle. Iba1 could be a DR marker, since inflammation is an outcome of this disease and Iba1 is found in activated macrophages in inflamed tissues (Ibrahim *et al.*, 2011; Ito *et al.*, 1998).

1.3.1.2.2. Cytokines

The inflammatory response also involves specific mediators like cytokines, interleukins and lymphokines in response to certain stimuli. In previous studies with samples collected from patients with Proliferative Diabetic Retinopathy, was demonstrated that pro-inflammatory cytokines, like interleukin-1β (IL-1β) and interleukin-8 (IL-8), are significantly increased (Elner *et al.*, 1995; Tang and Kern, 2011).

Interleukin 1β (IL-1β) is produced by caspase-1 and is associated with local inflammatory response and in hyperglycemia is increased in retinal Müller cells, which induces cell death and

vascular dysfunction (Mohr *et al.*, 2002; Vincent and Mohr, 2007). IL-1β associated with another cytokines is responsible for collagen and fibroblasts synthesis, resulting in proliferation and contraction, influencing angiogenic activity (Demircan *et al.*, 2006). Previous studies demonstrated that the use of antioxidants in the diet reduces the IL-1β expression promoted by diabetes and inhibits the degeneration of capillaries in animal models (Vincent and Mohr, 2007). The presence of IL-1β modulates the production, by epithelial cells, of the IL-8 (Muto *et al.*, 2015). IL-8 induce chemotaxis, the process of recruitment of immune cells to the site of damage promoting vascular permeability and angiogenesis (Elner *et al.*, 1995). IL-8 specifically promotes de attraction and activation of neutrophils that release enzymes that degrade connective tissues (Bickel M., 1993). Previous studies with epithelial cells stimulated with IL-1β have shown an increase in IL-8 secretion; while treatment with antioxidants successfully decreased its expression (Muto *et al.*, 2015).

1.3.1.3. Glucose content in RPE cells

The RPE transports water and electrolytes from the subretinal space to the choroid and, in the other direction, transports glucose from the blood to the photoreceptors. As glucose is the only fuel source of retinal cells, an adequate glucose delivery is crucial. The transport of glucose is exclusively mediated by a sodium-independent glucose transporter 1 (GLUT1, 55 kDa) located in the RPE basal membrane (Ban and Rizzolo, 2000; Bergersen *et al.*, 1999). GLUT1 adapts the glucose transport to different situations, like hypoxia, growth factors and glucose levels. Our group has shown that in DR, hyperglycemia and hypoxia directly affect GLUT1 expression (Calado *et al.*, 2016). As cells need a higher consumption of glucose in oxygen deprivation, the GLUT1 expression increases (Calado *et al.*, 2016) to achieve an effective glucose transport.

RPE cells secrete multiple essential trophic factors, among them PEDF (Araújo, Santos and Silva, 2018; Ponnalagu *et al.*, 2017). Previous studies from our group with a RPE cell line demonstrated that high levels of GLUT1 and, consequently an increase in glucose supply, has negative effects in PEDF expression and promotes the VEGF expression, contributing to the imbalance between these two proteins, promoting angiogenesis (Calado *et al.*, 2016). These results show that high levels of glucose directly affect the secretory function of RPE cells.

1.4. Phenolic metabolites

Polyphenols are molecules with aromatic rings and hydroxyl groups (-OH) in their structure, naturally found in fruits, vegetables, cereals and beverages, including tea, red wine, olive oil and berries. They are identified as secondary metabolites of plants, involved in its protection against reactive oxygen species (ROS) produced during photosynthesis. At the present, have been identified more than 8000 polyphenolic compounds in various plants species. Fruits and some beverages are the main source of polyphenols. In fruits there are a complex mixture of polyphenols dependent on environmental factors such time of harvest, sun exposure, processing and storage (Kroon *et al.*, 2004). Berries are an exceptional exogenous source of polyphenols, which makes them a good alternative for healthy diet to prevent the progression of diseases like DR.

The preventive or therapeutic action of these compounds are dependent of its bioavailability, meaning the proportion of nutrient that is digested, absorbed and metabolized (Pandey and Rizvi, 2009). In the first step and before absorption, the compounds are hydrolyzed by intestinal enzymes/colonic microflora and during absorption the polyphenols suffers methylation, sulfation and/or glucuronidation and reaches the blood and tissues in a different form from those present in food (Pandey and Rizvi, 2009). The intestinal absorption levels and the nature of the metabolite circulating in the plasma its directly related with the structure of the polyphenols. The metabolism of polyphenols starts as soon as the compounds enter the body, although some are rapidly absorbed others are not (Lafay and Gil-Izquierdo, 2008; Pimpão *et al.*, 2014).

The scientific interest on these compounds have increased based on their anti-inflammatory, anti-diabetic, anti-mutagenic, anti-aging and neuroprotective properties, which may have beneficial effects on human health (Figueira *et al.*, 2017; Pandey and Rizvi, 2009).

There are various studies on the anti-diabetic effects of polyphenols. These compounds influence the glycemia through different mechanisms like inhibition of glucose absorption in the gut or in peripheral tissues. Some studies show the inhibition of glucose transporters by polyphenols (Matsui *et al.*, 2001). For example, onion polyphenols have the capacity of protect diabetic patients from oxidative stress (Pandey and Rizvi, 2014) and polyphenols from vegetables act as potent anti-diabetic agents because they lowered the levels of blood glucose and increase plasma insulin (Barone, Calabrese and Mancuso, 2009; Eun *et al.*, 2007).

Oxidative stress is considered the main process behind neurodegenerative diseases. Since polyphenols have important anti-oxidative properties, their consumption may confer protection in neurological disorders because they influence important cell processes like signaling, apoptosis, proliferation and redox balance (Singh *et al.*, 2008). In fact, people who drink three to four glasses of wine per day had 80% decreased neurodegeneration compared to those who did not (Scarmeas N, Luchsinger JA, Mayeux R, Stern Y, 2009).

The combination of anti-inflammatory and anti-oxidant properties makes polyphenols good anti-aging compounds, since inflammation and oxidative stress are characteristic of aging process (Joseph, Shukitt-Hale and Casadesus, 2005). Supplementation of diet with spinach, strawberries and blueberries for 8 weeks were effective in reversing age-related markers in aged rats (Shukitt-Hale, Lau and Josep, 2008). Polyphenols are also effective in protecting brain from the adverse effects of aging since these compounds cross the blood-brain barrier (BBB), which controls the influx of nutrients and drugs in the brain (Figueira *et al.*, 2017).

The mechanisms of action of polyphenols in the human body are poorly known, so it is important to determine the compounds that can be found in the body and use them in relevant concentrations. Thus, bioavailability studies are very important and needed (Manach *et al.*, 2004; Rio, Del *et al.*, 2013).

Claúdia Santos and co-workers identified for the first time in human plasma the presence in high concentrations of sulfated metabolites of catechol (catechol-*O*-sulfate, Cat-sulf) and pyrogallol (pyrogallol-*O*-sulfate, Pyr-sul), after the ingestion of a purée containing five different berry fruits, suggesting that the sulfated form of the metabolites results from the polyphenols metabolism in the body (Pimpão *et al.*, 2015). Moreover, the same group identified, for the first time, Cat-sulf in significant concentrations in the urine of volunteers after the ingestion of the same purée (Pimpão *et al.*, 2014). Previously, in other study, it was identified Pyr-sulf in volunteers' urine after the ingestion of green and black tea (Daykin *et al.*, 2005; Dorsten, Van *et al.*, 2006; Hooft, Van Der *et al.*, 2012). The pyrogallol was found as a mixture of two isomers (pyrogallol-1-*O*-sulfate and pyrogallol-2-*O*-sulfate) in similar proportions, but *in vitro* studies suggests that pyrogallol-2-*O*-sulfate was the main form transported (Pimpão *et al.*, 2014, 2015). The structure of pyrogallol, catechol and the sulfated forms are represented in Figure 1.4.

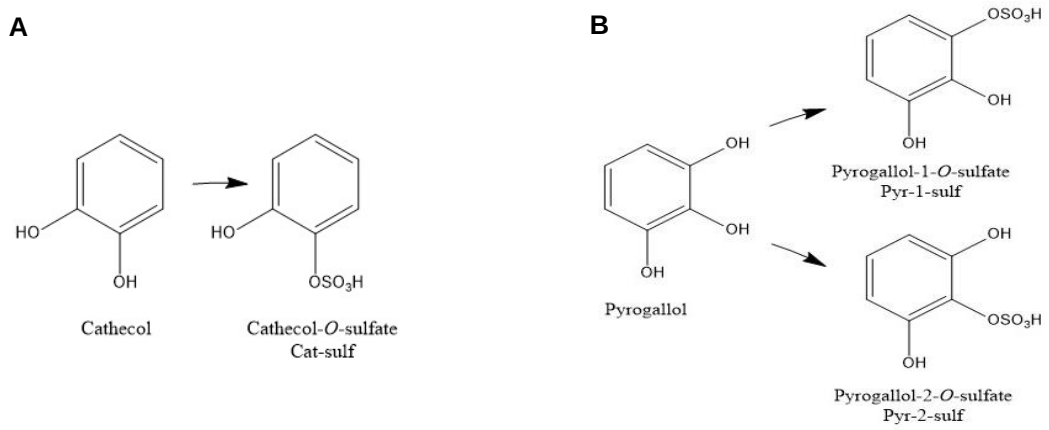

Figure 1.4 Principal bioavailable phenolic metabolites in human plasma and urine, after the ingestion of a purée containing five different berry fruits. (A) Reaction of sulfation of catechol results in the metabolite catechol-O-sulfate (Cat-sulf). (B) Reaction of sulfation of pyrogallol results in two compounds aproximately in similar proportion, pyrogallol-O-sulfate (Pyr-sulf).

The selective permeability across the BBB limit the bioavailability and protective effects of phenolic metabolites (Borges *et al.*, 2013; Chen *et al.*, 2015). A group of researchers have used a BBB *in vitro* model to confirm that phenolic metabolites could be transported through the BBB endothelium, and have shown that differences in endothelial transport is due to the metabolite chemical structure. However, it is not clear if the transport is mediated or if is by simple diffusion. The results suggest that the endothelial cells may favor the uptake of the most abundant bioavailable metabolite of a mixture in similar proportions, which confirm the detection of only one isomer of pyr-sulf (Figueira *et al.*, 2017; Pimpão *et al.*, 2015). The same group, show for the first time that the most abundant metabolite in circulation, Pyr-sulf, was also the most effective in prevent oxidative damage in *in vitro* studies with endothelial cells model (Pimpão *et al.*, 2014)

1.5. *In vitro* and *in vivo* models of Diabetic Retinopathy

The models used to study the mechanisms of disease are very important tools. Using *in vitro* models, we are able to control the conditions of the environment, spend less amounts of reagents and avoid animal sacrifice. However, *in vivo* models allow us to test a hypothesis under similar conditions to those detected in human. Despite *in vitro* models not fully mimicking the *in vivo*, they are less expensive, and results can be obtained faster.

As mentioned before, RPE can express and secrete several factors, which allows to confirm some of DR characteristics. VEGF and PEDF are among the most important factors secreted by RPE cells. Analyzing VEGF messenger RNA (mRNA) or protein expression together with PEDF after the onset of hyperglycemia and hypoxia, we can determine if there is an imbalance between these two factors. This imbalance is a hallmark of DR that can contributes to retinal neovascularization, which can be further confirmed with *in vivo* experiments. High glucose concentration and hypoxia conditions contributes to an inflammatory response, so the presence of specific cytokines in mRNA or protein expression analysis, both *in vitro* or *in vivo*, confirms the onset of inflammation, another important hallmark of DR (Araújo, Santos and Silva, 2018).

For this work we used two different models which will be described in sections 3.1. and 3.5.

2. Objectives

The prevalence of DM worldwide continues to increase and DR is the most common ocular complication of this disease in developed countries (Fong *et al.*, 2004). Oxidative stress, inflammation and abnormal angiogenesis are major hallmarks in DR. This disease can lead to vision loss and affects mostly working-age adults (Cheung, Mitchell and Wong, 2010). Therefore, it is extremely important develop efficient treatments for this pathology.

Over the years the beneficial effects in human health of polyphenols, compounds present in various foods, have been studied. Studies with berries showed relevant concentrations of two different phenolic metabolites (catechol-*O*-sulfate and pyrogallol-*O*-sulfate) in human plasma and urine (Pimpão *et al.*, 2014, 2015). Based on their anti-inflammatory, anti-diabetic, anti-mutagenic, anti-aging and neuroprotective properties, polyphenols can be used as treatment for several diseases (Figueira *et al.*, 2017).

The objective of this work is to evaluate the potential benefit of Pyr-sulf and Cat-sulf phenolic metabolites in the outcome of DR.

This study is divided in two specific aims:

1. The evaluation of the effects of the phenolic metabolites' treatment *in vitro* using RPE cells as model;

2. The administration of the phenolic metabolites and evaluation of their effects *in vivo* in a diabetic mouse model.

3. Materials and Methods

3.1. *In vitro* model: D407 RPE cell line

In vitro cell models can be either primary cultures or continuous cell lines of human origin. In the beginning, *in vitro* studies of human RPE cells has been done with primary cultures, but these cultures only survive eight to ten passages (Davis *et al.*, 1995). Thus, the need to create a continuous cell line was imperative. In 1995 a group of investigators extract cells from a globe of the eye of a dead 12-year-old boy, cultured it and they were able to maintain the cells in cultured for more than 200 passages (Davis *et al.*, 1995) . This cell line, named D407, is now considered a spontaneously transformed RPE cell line that retain their epithelial morphology and characteristics which makes it a great model to study alterations in different conditions (Davis *et al.*, 1995). However, D407 cells do not polarize in culture and they do not synthesize the characteristic pigment. In this work we have used D407 cells because our study is related with the eye metabolism, so we have used a RPE cell line that maintains a high degree of epithelial morphology is very important. We can use this cell line for a high number of passages and they maintain a morphology similar to early passages (Davis *et al.*, 1995). Furthermore, D407 cells maintain its cobblestone epithelial morphology unlike other cell lines whose pigment production ceases under the culture conditions and lose their morphology (Davis *et al.*, 1995).

3.1.1. Cell culture

For the *in vitro* studies were used D407 cells, RPE cell line (Davis *et al.*, 1995), kindly provided by Dr. Jean Bennett (University of Pennsylvania, USA). The cells grown in 25 cm^2 flasks (Orange Scientific, Belgium) containing Dulbecco's Modified Eagle's Medium (DMEM) with 5.5 mM D-glucose (GE Healthcare, USA) supplemented with 1% penicillin/streptomycin (Sigma-Aldrich, USA) and 5% fetal bovine serum (FBS) (Sigma-Aldrich, USA) and kept in a humidified chamber at 37ºC with 5% CO_2. The medium was changed every 2 days. Standard culture protocols suggest growing cells in high glucose media; however, we have cultured the cells in DMEM with 5.5 mM D-glucose (non-diabetic conditions) and we have shown no deleterious effects on cell viability or function.

After reaching confluence (70%-90%), cells were washed with PBS 1x and detached with trypsin-EDTA at 37ºC. After 5 minutes DMEM with 5.5 mM D-glucose was added to the flask and the final volume were centrifugated (1 min, 1500 rpm). The pellet was resuspended in the fresh medium.

3.1.2. Polyphenol treatment

To test the effect of the polyphenol's metabolites on D407 cell line, cells were seeded at a density of $3x10^5$ cell/well and after 24 h of incubation at 37ºC, the medium was changed. Cells were cultured for 24 h in DMEM with 5.5 mM of D-glucose to mimic a physiological concentration, in DMEM with 25 mM of D-glucose, to mimic a pathological condition (hyperglycemia) and in DMEM with 5.5 mM of D-glucose containing mannitol with a final concentration of 25 mM, as an osmolarity control. Mannitol (AppliChem, Germany) was chosen because it has no known biological activity and cannot be used by the cells as energy source (Duffy *et al.*, 2006).

The polyphenol compound (Cathecol-*O*-sulfate C_f = 5 µM or Pyrogallol-*O*-sulfate C_f = 6.5 µM), kindly provided by Claúdia Santos (ITQB/iBET), was incubated for 24 h in the three different media without FBS. To induce hypoxia cells were incubated with desferrioxamine (DFO) (Sigma-Aldrich, USA) at a final concentration of 100 µM (Aprelikova *et al.*, 2004; Wu and Yotnda, 2011), for 16 h or 24 h. All the protocol is represented below in Figure 3.1.

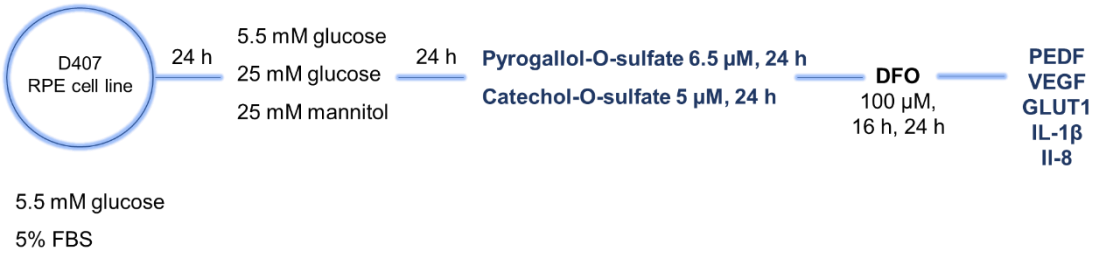

Figure 3.1 Schematic representation of the in vitro setup for testing the phenolic metabolites.

3.2. MTT cell viability assay

MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) is a yellow substrate that, when metabolized by the cells turns to dark blue, a formazan product. So, the metabolic activity of D407 cells is directly proportional to the quantity of formazan formed (Berridge, Herst e Tan, 2005).

D407 cells were seeded at a density of $2x10^4$ cell/well in a 48-well plate, for a final volume of 500 µl/well of DMEM with 5.5 mM medium (supplemented with 1% penicillin/streptomycin and 5% FBS), the standard condition. After 24 h the media was changed, and cells were exposed to different media conditions as previously described in 3.1.2. Treatment with the polyphenolic metabolite compound (Cathecol-*O*-sulfate C_f=5 µM or Pyrogallol-*O*-sulfate C_f=6.5 µM) was maintained for 24 h and hypoxia induced for 24 h or 16 h with DFO at a final concentration of 100 µM.

In each well 25 µL of MTT at a final concentration of 0.25 mg/ml (Sigma-Aldrich, Missouri, USA) was incubated at 37ºC for 3 hours. At the end of the incubation period, the formazan was

dissolved with 250 µl of 0.04 N HCl (Sigma-Aldrich, USA) in isopropanol 100% (Fisher Scientific, USA) and the absorbance was measured in a Biotrak II Plate reader (Amersham Biosciences, UK) at 540/620 nm.

3.3. Quantitative Real Time PCR (RT-qPCR)

3.3.1. RNA extraction

To promote the lysis of the cultured cells, TRIzol™ Reagent (Sigma-Aldrich, Missouri, USA) were added (300 µl) to each well. After homogenization, all the volume was transferred to a previously identified tube and incubated 2-3 minutes with chloroform (VWR, USA) to precipitate the deoxyribonucleic acid (DNA). After centrifugation (12000 g, 4ºC, 15 min), the aqueous part, containing the ribonucleic acid (RNA), was carefully transferred to a new tube with isopropanol for 10 minutes. The samples were centrifugated (12 000 g, 4ºC, 10 min) and the supernatant removed. A solution of 75% ethanol (Sigma-Aldrich, USA) was used to wash the RNA and the sample centrifugated again (7500 g, 4ºC, 5 min). In the end, the pellet was resuspended in 20 µl of RNAse-free water (Sigma-Aldrich, USA).

To evaluate the quality and quantity of the RNA, samples were analyzed in a NanoDrop™ 2000 (Thermo Scientific, USA) spectrophotometer.

3.3.2. cDNA synthesis

The complement deoxyribonucleic acid (cDNA) was synthetized with a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA). The RNA extract was diluted to a ratio of 1000 ng of RNA per 10 µl of RNAse-free water. To perform the reaction, each tube had a final volume of 20 µl (10 µl of the sample and 10 µl of the master mix). The reactions were done in a MyCycler™ Thermocycler (BioRad, USA).

3.3.3. RT-qPCR

This technique was used to analyze the expression of PEDF, VEGF, IL-8, IL-1β genes and β-Actin gene as a control. To perform the RT-qPCR, we used the cDNA obtained, prepared a mix with RNase-free water, containing the correspondent primers (Sigma-Aldrich, USA), forward and reverse, and Ssofast Evagreen Supermix (Bio-Rad, USA). In a 96 well-plate, 13 µl of the Mix and 2 µl of the diluted cDNA sample were added, for a final volume of 15 µl. The reactions were done in a 7300 real Time PCR System (Applied Biosystems).

The primers sequence and annealing temperature are listed in Table 3.1. For the PCR reactions, the conditions used were: denaturation at 95ºC for 1 minute, followed by 40 cycles of denaturation at 95ºC for 15 seconds and annealing for 15 seconds at the gene-specific temperature (Table 3.1), the extension step was done at 65ºC for 30 seconds. The β-actin was

used has housekeeping gene, to normalize the mRNA levels of PEDF, VEGF, IL-8 and IL-1β. For mRNA quantification we used the comparative method represented in Equation 3.1 (Schmittgen and Livak, 2008).

$$relative\ expression = 2^{-\Delta\Delta CT}$$

$$\Delta\Delta CT = [C_{T\,(gene\ of\ interest\ )} - C_{T\,(\beta-actin\ )}]_{Treated\ \ sample\ } - [C_{T\,(gene\ of\ interest\ )} - C_{T\,(\beta-actin\ )}]_{Untreated\ \ sample}$$

Equation 3.1 Comparative Method

Table 3.1 Primers used in RT-qPCR

Gene	Primer sequence	Annealing temperature (ºC)
hPEDF	FW 5' CGACCAACGTGCTCCTGTCT 3'	63.3
	RV 5' GATGTCTGGGCTGCTGATCA 3'	
hVEGF	FW 5' ACTTCTGGGCTGTTCTCG 3'	72.3
	RV 5' TCCTCTTCCTTCTCTTCTTCC 3'	
hIL-8	FW 5' ATAAAGACATACTCCAAACCTTTCCAC 3'	62.3
	RV 5' AAGCTTTACAATAATTTCTGTGTTGGC 3'	
hIL-1β	FW 5' AAATACCTGTGGCCTTGGGC3'	62.3
	RV 5' TTTGGGATCTACACTCTCCAGCT 3'	
hβ-Actin	FW 5' GCAAAGACCTGTACGCCAAC 3'	59
	RV 5'AGTACTTGCGCTCAGGAGGA 3'	

FW – forward; RV - reverse

3.4. Western blot

3.4.1. D407 protein extraction

To obtain cellular lysates, cells were washed with PBS 1x, and collected with a cell scraper in cold RIPA buffer (50 mM Tris-HCl pH 7.4, 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl and 1 mM EDTA) with protease inhibitor cocktail 1x (Sigma-Aldrich, USA). The samples were transferred to tubes properly identified, incubated on ice for 20 minutes, centrifugated (13 200 rpm, 4ºC, 20 min) and the supernatant was collected. The samples were stored at -20ºC.

3.4.2. Protein quantification

The protein quantification was done by the Bradford Assay, using BSA (bovine albumin serum, NZYTech, Portugal) as standard and protein assay dye reagent concentrate (BioRad, USA). The standard concentrations of BSA (1 mg/mL; 0.5 mg/mL; 0.25 mg/mL; 0.125 mg/mL and 0.0625 mg/mL) and the dye reagent were prepared in ultrapure H_2O. The cellular lysates were diluted in ultrapure H_2O (1:5). In a 96-well plate, the cell and standard samples were loaded, and the dye reagent was added to each well. The absorbance was read in a Biotrak II Plate reader at 590 nm. The concentration of protein was exactly determined by linear regression.

3.4.3. Western blot

The samples were diluted in RIPA with 4x Laemmli Sample Buffer (Bio-Rad, USA) and heated at 95ºC for 5 minutes for DNA denaturation. Then the samples were loaded on a 12% SDS polyacrylamide gels along with 5 µl of a PageRuler Plus Prestained Protein Ladder 2x (Fisher Scientific, USA). Electrophoresis was performed at 100-120V in electrophoresis buffer (25 mM Tris-HCl, 192 mM Glycine and 0.1% SDS) until the dye reaches the bottom of the gel.

Proteins were transferred to polyvinylidene fluoride membrane (GE Healthcare, UK), previously activated for 20 seconds with methanol, following deionized H_2O for 20 seconds and cold transfer buffer (0.192 M glycine, 0.025 M Tris pH 8.3, 10% methanol) for 5 minutes, in the TRANS-BLOT SD equipment (Bio-Rad, USA) for 20 minutes at 25 V.

To block non-specific binding sites, the membrane was covered with 5% BSA in TBS-T (Tris-buffered saline 0.1% Tween-20) (Sigma-Aldrich, USA) for 72 h at 4ºC, with gently agitation. The appropriate primary antibody for the protein of interest was incubated overnight at 4ºC, with gently agitation, diluted in 5% BSA in TBS-T.

After washing with TBS-T three times for 5 minutes, the correspondent secondary antibody diluted in 5% BSA TBS-T was incubated for 1 hour at room temperature, with gently agitation. The dilutions used for each antibody are listed in Table 3.2. β-actin (Sigma-Aldrich, USA, 1:5000 dilution), incubated for 1h at room temperature, was used as loading control. The membrane was then washed for 5 minutes each with TBS-T and incubated 5 minutes with ECL

Prime Western Blotting Detection Reagent (1:1) (GE Healthcare, UK) for visualization in a ChemiDoc™ imaging System by Bio Rad.

For stripping, the membrane was washed three times with TBS-T for 5 minutes, incubated for 5 minutes with Sodium Hidroxyde (Sigma-Aldrich, USA) 2N, followed by a TBS-T three times wash for 5 minutes and incubated with the new primary antibody.

Table 3.2 Primary and secondary antibodies used for immunoblotting.

Antibody		Brand	Dilution
Primary	Rabbit polyclonal anti-PEDF	Santa Cruz Biotechnology, USA	1:500
	Rabbit polyclonal anti-VEGF	Abcam, UK	1:1000
	Rabbit polyclonal anti-GLUT1	Santa Cruz Biotechnology, USA	1:3000
	Goat polyclonal anti-Iba1	Abcam, UK	1:500
	Mouse monoclonal anti-β-actin	Sigma- Aldrich™, USA	1:5000
Secondary	HRP-conjugated goat anti-rabbit IgG	Santa Cruz Biotechnology, USA	1:5000
	HRP-conjugated goat anti-mouse IgG	Santa Cruz, Biotechnology, USA	1:5000
	HRP-conjugated donkey anti-goat IgG	Santa Cruz, Biotechnology, USA	1:5000

3.5. *In vivo* model: Ins2^{Akita} mice

There are several animal models to study complications associated with DM, like rodents, dogs, pigs or primates, but mouse and rat are the most used because of their small sizes and short lifespan (Olivares *et al.*, 2017).

Since DR is a complex disease with both genetic and environmental influences, diabetes in animal models can be pharmacologically induced or by genetic modification. There are different methods to induce diabetes: surgical removal of the pancreas, drug administration, high sugar diets and for features of DR, direct laser or chemical damage in the eye (Jo *et al.*, 2013; Olivares *et al.*, 2017). The genetic modified models include strain-specific, spontaneous and genetically edited mutations. There are at least five different genetic mouse models of DR, that vary in progression and pathology of disease.

In this work, we have used Ins2^{Akita} mouse, a spontaneous model of Type I DM, that results from a mutation in the mouse insulin II gene (Ins2), leading to insulin protein misfolding. The abnormal protein accumulates in pancreatic β-cells resulting in its death and consequently in a decrease in insulin secretion and hyperglycemia. Up to 8 months of age (Cai and McGinnis, 2016; Jo *et al.*, 2013; Olivares *et al.*, 2017) these mice show several characteristics that provide the opportunity to study the pathophysiology of DR and gives it advantage over other animal models. First, the mice breed well and are fertile. Second, they have a stable insulin-deficient diabetes and can be maintained without an exogenous insulin source. Third, the onset of diabetes does not involve immunologic alterations (Barber *et al.*, 2005). In previous studies researchers have tested Ins2^{Akita} as a model of early DR. They have shown in these mice a loss

of the blood-retinal barrier function, an increase in vascular permeability, and a thickening of the vascular basement membrane. Furthermore, the number of leukocyte adherent to the vascular wall was significantly elevated and microglia is activated, which confirm that the inflammation component of DR is present in these mice (Barber *et al.*, 2005; Gastinger *et al.*, 2008). All these features makes this model an excellent tool to study the progression of DR and neuroprotective treatments (Robinson *et al.*, 2012).

With $Ins2^{Akita}$ we can evaluate the expression of proteins expressed and secreted by all the retina cells, unlike the *in vitro* model used in this work, where we can only analyze proteins expressed and secreted by RPE. For example, Iba1 is secreted by microglia cells. The presence of these biomarkers in mRNA or protein expression analysis of the mice retina confirm the inflammation state, an important characteristic of DR (Ibrahim *et al.*, 2011).

Previous studies with $Ins2^{Akita}$ mice demonstrated that advanced characteristics of DR, such as neovascularization and thinning, only develop at 6 months of age (Mclenachan *et al.*, 2013). Using these *in vitro* and *in vivo* models, we can study the hallmarks of early and advanced stages of DR and perform experiments to find an efficient treatment to this complication associated with DM.

3.5.1. Housing

To test the treatment *in vivo*, were used male C57Bl/6 $Ins2^{Akita}$ (diabetic) heterozygote mice as DR model and C57BL/6 age-matched (wild-type) as a control group (The Jackson Laboratory, USA). The animals were sacrificed at 8 and 9 months of age.

Mice were housed in individually ventilated cages, under controlled temperature, with continuous access to food and water on a 12 hours dark/light cycle.

To confirm the diabetic phenotype, the blood glucose levels were measured 2 months after birth in a drop of blood from a tail's cut, with reactive glucose strips (Contour Next, Ascencia Diabetes Care, Portugal). Animals exhibiting blood glucose ≥ 250 mg/dl were considered diabetic (Barber *et al.*, 2005).

3.5.2. Anesthesia

To anesthetize the animals, Avertin [2,2,2-tribromoethyl alcohol (Sigma-Aldrich, USA) with 2-methyl-2-butanol (Sigma-Aldrich, USA)] was dissolved in deionized water, heated up to 30ºC using a magnetic stirrer. The mixture was then filtered/sterilized through 0.2-micro filter. The preparation was stored at 4ºC and protected from light for up to 1 week.

3.5.3. Treatment with Pyr-sulf in C57BL/$Ins2^{Akita}$ mice

All the methods with animals were performed according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

The animals were anesthetized with Avertin (0.5 ml/25 g) and the cornea was superficially anesthetized with Anestocil (Edol, Portugal) as an eye drop. The pupil was dilated with Cicloplegicedol (Edol, Portugal). The immobilized animal was subretinal injected (Figure 3.2), with 2 µl of Pyr-sulf (6.5 µM) in the left eye, using a Hamilton Syr 10 µl 701 RN injector. The non-injected eye was used as control. After the injection gentamicin and dexamethasone were applied in the injected eye to reduce the risk of infection.

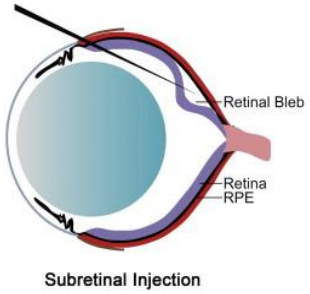

Figure 3.2 Subretinal injection. Its the most efficent method of injection to the RPE but is also very invasive (Koirala, Conley and Naash, 2013).

3.5.4. Retina extraction

Two weeks after Pyr-sulf injection, the animals are humanely sacrificed by cervical dislocation and the eyes removed. The retina was dissected and homogenized in ice-cold RIPA buffer using a motor pestle. After 20 minutes in ice the extracts were centrifugated (20 minutes, 4ºC, 13 200 rpm). The supernatant was collected and quantified by Braford method, as previously described in section 3.4.2, with a dilution of 1:10, for Western blot. The samples were stored at -80ºC.

3.6. Statistical analysis

Arithmetic means are given with standard error of the mean (SEM). Statistical analysis was performed using an unpaired t-test and two-way analysis of variance followed by the Sidak's or Tukey's Multiple Comparison test for multiple comparisons. A value of $p < 0.05$ was considered to be statistically significant.

4. Results and discussion

4.1. Differential effect of Cat-sulf and Pyr-sulf in the viability of RPE cells

To evaluate whether the phenolic metabolites influence the viability of the cells used for the *in vitro* tests, we have performed a MTT assay, an indirect method to evaluate cell viability. MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) is a yellow substrate that when metabolized by the cells turns to dark blue, a formazan product. This reaction and readout is only possible in living cells, so the cell viability is directly proportional to the quantity of formazan formed (Berridge, Herst and Tan, 2005). D407 cells were incubated with Cat-sulf 5 µM or Pyr-sulf 6.5 µM for 24h, as described in section in 3.1.1.

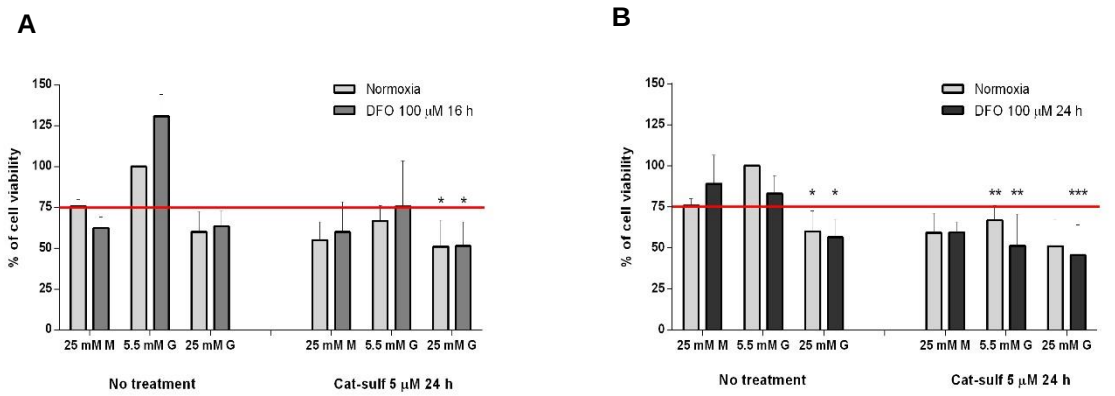

Figure 4.1 Cat-sulf treatment affects D407 cell viability. Effect of Cat-sulf (5 µM, 24 h), on RPE cell line viability under high glucose (25 mM glucose) and hypoxia (DFO 100 µM 16 h (A) or DFO 100 µM 24 h (B)) conditions, assessed by MTT assay. Values are expressed as percentage of control. N=4, *p < 0.05, ** p < 0.01 and *** p < 0.001 are significantly different from control (5.5 mM glucose, under normoxia with no treatment), determined by Sidak's (**A**) and Tukey's (**B**) multiple comparisons tests. M, Mannitol (osmolarity control) and G, Glucose.

Figure 4.1 shows significantly differences in cell viability after treatment of D407 cells with Cat-sulf, when compared with no treatment conditions. The percentage of cell viability decreases to about 75% (red line), suggesting that Cat-sulf affects the metabolic activity of D407 cells. The MTT is a reagent that is reduced to blue formazan crystals by mitochondrial succinate dehydrogenase (WANG *et al.*, 1996) and the color intensity of the formazan is proportional to the number of viable cells (O'Toole *et al.*, 2003). However, some phytochemicals may direct interact with MTT or alters the activity of succinate dehydrogenase (Devika and Stanely Mainzen Prince, 2008; Hsu *et al.*, 2003).

Some studies with green tea polyphenols show that these compounds may interfere with formazan formation (Bruggisser *et al.*, 2002; Maioli *et al.*, 2009). When compared with other viability tests, the MTT was the only one with different results (Wang, Henning and Heber,

2010). In fact, the results obtained after cell exposure to Cat-sulf may be due to its reaction with formazan, which affects cell metabolic activity. To corroborate these results we must perform other viability tests, such as Flux Cytometry, the traditional Trypan Blue dye exclusion assay, the ATP-based method using luciferin-luciferinase reaction to produce bioluminescence or a DNA-based assay kit to produce strong fluorescence of nucleic acid (Chen and Cushion, 1994; Jones *et al.*, 2001; Kanemura *et al.*, 2002).

Contrary to the results obtained with Cat-sulf, the treatment with Pyr-sulf does not influence the viability of D407 cells (Figure 4.2).

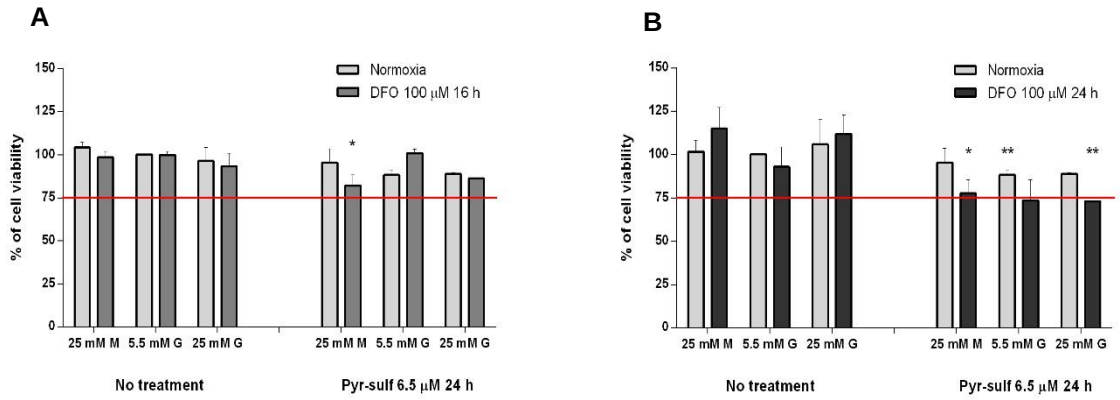

Figure 4.2 Pyr-sulf treatment does not affect D407 cell viability. Effect of Pyr-sulf (6.5 µM 24 h), assessed by MTT assay, on RPE cell line viability under high glucose (25 mM glucose) and hypoxia (DFO 100 µM 16 h (A) or DFO 100 µM 24 h (B)) conditions. Values are expressed as percentage of control. N=4, *p < 0.05, ** *p* < 0.01 are significantly different from control (5.5 mM glucose, under normoxia with no treatment), determined by Tukey's multiple comparisons tests. M, Mannitol (osmolarity control) and G, Glucose.

These results may suggest that Pyr-sulf does not interfere with formazan formation in the same way as Cat-sulf, perhaps because of their chemical structures the compounds are metabolized differently by cells. In previous studies, it was shown that Pyr-sulf can be metabolized in two different ways. One of them suggests that Pyr-sulf can be metabolized in Cat-sulf (Figueira *et al.*, 2017). This transformation can explain the results obtained after cell exposure with Pyr-sulf in the MTT assay. Also, the synthetic Pyr-sulf used for this assay is a mixture in equal proportions of two isoforms of the compound (Pyr-*O*-sulf 1 and Pyr-*O*-sulf 2) which, in terms of metabolization, can be more time consuming than that of the Cat-sulf and therefore does not affect the cellular viability in 24 h of treatment. MTT reagent itself can be cytotoxic and if the metabolization of the compound ends before the period of incubation of MTT, then the cells are left with the reagent and their viability may be affected. In the future we could test a longer period of incubation with Pyr-sulf and assess cell viability to confirm this hypothesis. Also, D407 cells treated with Cat-sulf were, in general, older than the cells exposed to Pyr-sulf which presupposes different metabolic activities. Moreover, different results observed for both metabolites may result from technical problems, especially during the removal of cell

culture medium before dissolving the formazan crystals. This is achieved by aspiration with a vacuum bomb, leading to possible removal of crystals and, therefore, inconsistent results between independent experiments.

In addition, we also used the MTT assay to assess the time of cell exposure to hypoxia. To perform this study, we perform the MTT assay with two different hypoxia timepoints, 16 h and 24 h. Our results show that after 24 h of exposure to DFO the cell viability is lower than with 16 h of hypoxia for both phenolic metabolites (Figure 4.1and Figure 4.2). Therefore, based on results previously obtained in our laboratory and according with the literature (Aprelikova *et al.*, 2004), in the following experiments we decided to incubate the DFO for 16 h and not for 24 h to be sure that the metabolic activity of the cells is not compromised due to hypoxia.

After the optimization of the protocol, we decided to perform the *in vitro* studies only with Pyr-sulf 6.5 µM and to induce hypoxia for 16 h. The cells were cultured as previously described (section 3.1.1) and after 24 h of treatment, the protein or RNA was extracted for Western blot or RT-qPCR, respectively.

4.2. Treatment with Pyr-sulf alters the expression of pro- and anti-angiogenic factors

As previously reported, in DR there is an imbalance between pro- and anti-angiogenic proteins, such as PEDF and VEGF. The levels of the pro-angiogenic factor (VEGF) are abnormally increased and the anti-angiogenic protein (PEDF) levels decreased (Farjo and Ma, 2010).

To evaluate if the treatment with Pyr-sulf has beneficial effects in balancing these protein levels, we have developed an *in vitro* system to simulate DR in RPE cells, where D407 cells were cultured in low glucose (5.5 mM D-Glucose) followed by high glucose (25 mM D-Glucose) conditions and in normoxia and hypoxia (DFO 100 µM 16 h). The cells were incubated with Pyr-sulf for 24 h in free-serum medium. To analyze protein levels and mRNA expression we perform a Western blot or RT-qPCR, respectively, and the results are represented below.

Figure 4.3 represents the levels of gene (A) and protein (B) expression of PEDF with and without treatment with Pyr-sulf for 24 h.

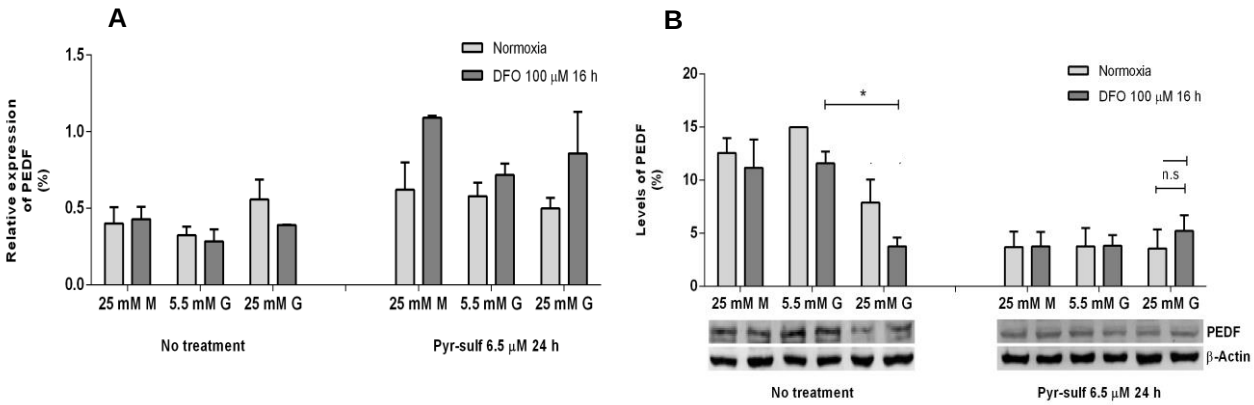

Figure 4.3 Pyr-sulf treatment alters PEDF mRNA expression and levels of protein under diabetic conditions. (A) RT-qPCR analysis of PEDF mRNA expression in D407 cells after Pyr-sulf treatment (6.5 μM 24 h). β-Actin was used as housekeeping control, N=4. (B) Western blot analysis of PEDF protein levels in D407 cells after treatment with Pyr-sulf (6.5 μM 24 h). Quantitative data normalized by the intensity of β-Actin. N=5. *$p < 0.05$ is significantly different from 5.5 mM glucose under hypoxia; n.s indicates no significative differences between conditions, determined by Tukey's multiple comparisons tests. M, Mannitol (osmolarity control) and G, Glucose.

Regarding the gene expression of PEDF, conditions without treatment tend to express less PEDF in hypoxia, compared with normoxia (Figure 4.3 A), especially when cells are in high glucose medium. This is in accordance with what is described for DR, with oxygen deprivation leading to PEDF downregulation (Cheung, Mitchell and Wong, 2010; Fong *et al.*, 2004). After treatment with Pyr-sulf the PEDF relative expression increases in hypoxic conditions compared with those without treatment, which suggest that this phenolic metabolite influences PEDF expression at mRNA levels. Although not statistically significant, we observe an increase in mRNA relative expression of PEDF when treated cells are in high glucose (25 mM G) under hypoxia, compared with the high glucose (25 mM G) in normoxia condition. These results may suggest a beneficial effect of treatment with Pyr-sulf to re-establish the normal regulation of this anti-angiogenic factor.

In Figure 4.3 B, we observe that the levels of PEDF protein decrease when conditions without Pyr-sulf treatment are in hypoxia, which is accordance with the literature (Fong *et al.*, 2004) and with the results obtained for mRNA (Figure 4.3 A). For the same untreated cells there is a significant decrease in PEDF protein levels when cells are in high glucose (25 mM G) under hypoxia compared with untreated cells in low glucose (5.5 mM G) under hypoxia. These results are in accordance with the physiological situation (Araújo, Santos and Silva, 2018; Fong *et al.*, 2004), and corroborates previous work from our lab (Calado *et al.*, 2016), where the levels of PEDF are described to be reduced with the increase of glucose levels. However, after treatment with Pyr-sulf the PEDF protein levels decrease in all conditions compared with no treatment conditions. Nevertheless, treated cells in high glucose (25 mM G) under hypoxia shows a slight increase in PEDF levels when compared with the same cells in normoxia or low glucose (5.5 mM G) treated cells under hypoxia.

PEDF is a protein secreted by RPE cells. In order to complement the obtained results with cell lysates, we can perform studies to evaluate the expression of the protein secreted in the cell medium. There are some problems related with analyzing secreted proteins, for example low concentration of protein in the media, the contamination of these proteins with proteins released by cell lysis and contamination with proteins from the serum (FBS) (Chevallet *et al.*, 2007). To avoid these issues, we can perform an ELISA (Enzyme-Linked Immunosorbent Assay) to analyse the levels of secreted PEDF. This assay is a specific technique based on antigen-antibody interaction. In a 96-well plate the antigens present in the media sample are attached to the surface. Then we apply a specific antibody for PEDF, over the surface to bind to the antigen. The primary antibody can be detected by a secondary antibody linked to an enzyme, followed by a substrate to produce a visible signal. Between each step, the plate is washed to remove non-specific proteins and antibodies (Leng *et al.*, 2008). Another technique we can use is Immunoprecipitation. An antibody is also immobilized to a beaded support (agarose or magnetic beads) and then incubated with the sample containing the target protein. The target antigen will bind specifically to the specific antibody. This complex lysate is isolated from the support and analyzed by Western blot or other quantitative assay methods (Kaboord and Perr, 2008).

Figure 4.4 represents the levels of gene (A) and protein (B) expression of VEGF with and without treatment with Pyr-sulf for 24 h.

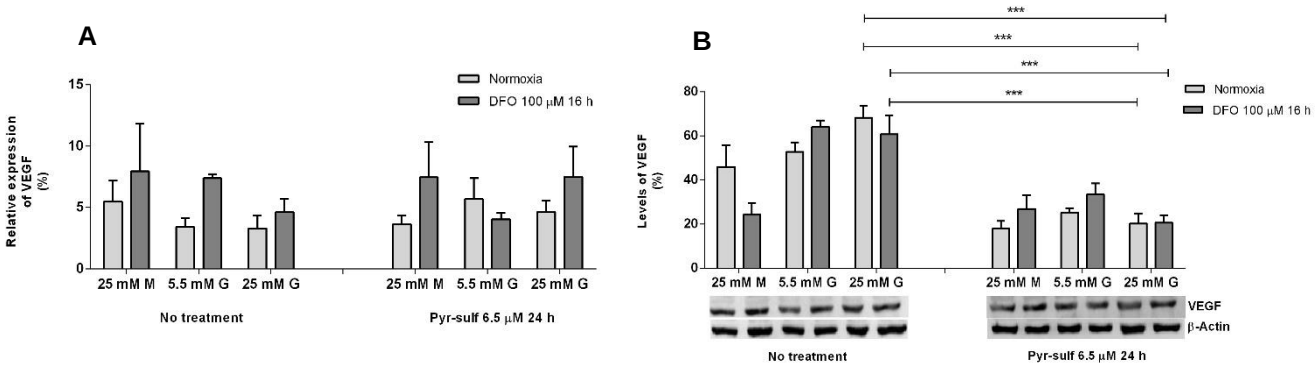

Figure 4.4 Pyr-sulf treatment decreases pro-angiogenic protein expression under diabetic environment. (A) RT-qPCR analysis of VEGF mRNA expression in D407 cells after Pyr-sulf treatment (6.5 µM 24 h). β-Actin was used as housekeeping control, N=4. (B) Western blot analysis of VEGF protein levels in D407 cells after the treatment with Pyr-sulf (6.5 µM 24 h). Quantitative data normalized by the intensity of β-Actin. N=5. ***$p < 0.001$ are significantly different from 25 mM glucose under normoxia or hypoxia with no treatment, determined by Tukey's multiple comparisons tests. M, Mannitol (osmolarity control) and G, Glucose.

It is well known that mRNA expression of VEGF increase when cells are under hypoxia (Cheung, Mitchell and Wong, 2010; Simão *et al.*, 2016), which is what we observe in the no treatment conditions (Figure 4.4 A). This increase is expected in hypoxia because cells need an increased blood supply, and VEGF promotes the formation of new blood vessels to guarantee cell survival. However, the decrease in VEGF mRNA in non-treated cells in high glucose (25 mM G) under hypoxia, compared with cells in low glucose (5.5 mM G) under hypoxia, is opposite what we were expecting (Figure 4.4 A). Support to our results can be found in the work of Benjamin and co-workers. (Benjamin, 2001), in which when cells are in high glucose medium a higher increase in VEGF mRNA expression is expected, because this factor controls the glucose passage to the retina (Benjamin, 2001). We hypothesized that there are at least two possible explanations for the decrease of VEGF: firstly, VEGF in hypoxia is secreted by RPE to the supernatant (Bian, Elner and Elner, 2007), and we are analyzing cell lysates and not the medium, explaining the decrease in VEGF expression. Secondly, as described in previous studies (Takenaka *et al.*, 2005), PEDF may block the VEGF production, which corroborates the decrease in VEGF mRNA expression in diabetic conditions .

Unfortunately, treatment with Pyr-sulf does not alter the expression profile when cells are in high glucose medium, which shows that Pyr-sulf has no influence in decreasing mRNA expression of VEGF in a pathological condition. On the other hand, when cells are in low glucose (5.5 mM G) under hypoxia, treatment with Pyr-sulf tends to decrease the mRNA expression of VEGF compared with non-treated cells in the same conditions. This result opens an opportunity to explore the Pyr-sulf potential as preventive therapeutic agent. Pyr-sulf may help to prevent new blood vessels formation and other hallmarks of DR, by avoiding pro-angiogenic protein mRNA overexpression.

Similarly, to what we did with PEDF, we have analyzed VEGF protein expression (Figure 4.4 B). In cells without treatment we observe a slight increase in VEGF levels in low glucose (5.5 mM G) when comparing hypoxia with normoxia. In untreated cells cultured in high glucose (25 mM G) there is a slight increase in VEGF protein compared with the cells cultured in low glucose (5.5 mM G) medium under normoxia, despite the decrease at mRNA levels. In fact, it was already described that high glucose affects VEGF expression at protein level by Chang *et al.*, 2018. Also, these results are similar to those previously obtained in our lab. Overall, the levels of VEGF significantly decrease after the treatment with Pyr-sulf. In diabetic conditions (25mM G under hypoxia) the levels of this pro-angiogenic protein are significantly lower after treatment when compared with the same condition without treatment, showing a beneficial effect of Pyr-sulf at protein level. There is also a significant decrease in VEGF levels in high glucose (25 mM G) normoxia condition between treated and non-treated cells. Contrary to what happen with PEDF, Pyr-sulf seems to influence VEGF expression at pos-translational levels.

4.3. Treatment with Pyr-sulf reduces the expression of the glucose transporter, GLUT1

Glucose is the only fuel source of retinal cells and it must be adequately delivered to supply all needs. GLUT1 is the only glucose transporter in the RPE membrane and has the capacity of adapt the transport in different situations (Sone, Deo and Kumagai, 2018). GLUT1 is able to increase the glucose transport in oxygen deprivation conditions, showing a direct effect of hypoxia in this glucose transporter (Calado *et al.*, 2016). Furthermore, an increase in glucose levels leads to a significant increase in GLUT1 protein, showing the effect of glucose concentration in GLUT1 protein expression (Calado *et al.*, 2016). Since hypoxia and hyperglycemia are hallmarks of DR (Cheung, Mitchell and Wong, 2010), GLUT1 expression is directly affected in this disease (Calado *et al.*, 2016). Knowing that we intend to study the effect of Pyr-sulf in GLUT1 protein expression using an *in vitro* approach with D407 cells.

Figure 4.5 represents the levels of protein expression of GLUT1 with and without treatment with Pyr-sulf.

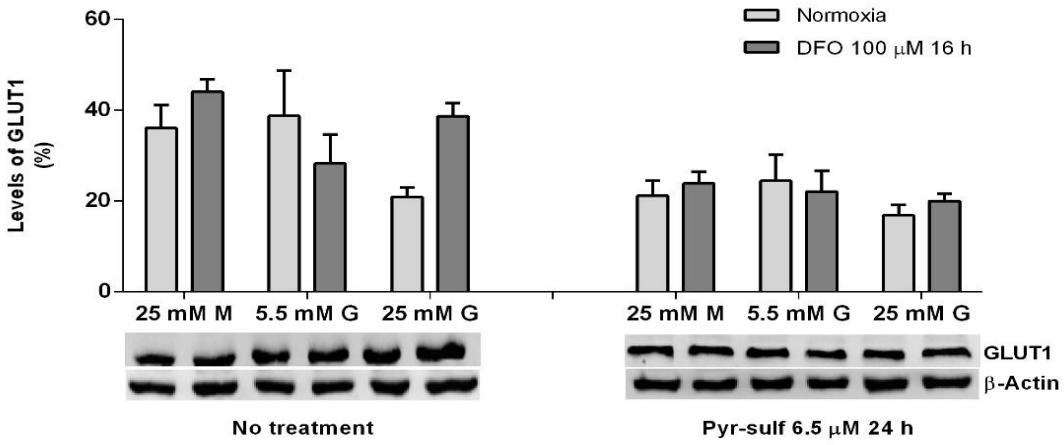

Figure 4.5 GLUT1 expression is reduced after treatment under diabetic conditions. Western blot analysis of GLUT1 protein levels in D407 cells after treatment with Pyr-sulf (6.5 μM 24 h).Quantitative data normalized by the intensity of β-Actin. N=5. M, Mannitol (osmolarity control) and G, Glucose.

The Western blot analysis show that expression of GLUT1 in cells without treatment in low glucose (5.5 mM G) under normoxia had no differences compared to cells under hypoxia. However, untreated cells in high glucose condition (25 mM G) under hypoxia shows an increased at protein level compared with the cells cultured in normoxia, showing the effect of glucose concentration in GLUT1, as well as the effect of oxygen privation.

After treatment with Pyr-sulf, GLUT1 levels decreases in all conditions compared to untreated cells. The protein expression is reduced in cells in high glucose (25 mM G) condition under hypoxia when compared to those without treatment, suggesting that there is a tendency, although not statiscally significant, for treatment with Pyr-sulf decrease the expression of GLUT1 protein, highlighting the beneficial effect of Pyr-sulf in controlling the transport of glucose in DR. To confirm this hypothesis, we can perform a glucose consumption assay in the future, to evaluate the uptake of glucose by RPE cells and if it is affected by treatment with Pyr-sulf.

4.4. Pyr-sulf has anti-inflammatory effect in D407 RPE cells

As previously reported, phenolic metabolites have, among others, the important capacity of acting against inflammation. In DR, the hyperglycemia and hypoxia contribute to a chronic inflammatory state, since they promote microglia constant activation. In this situation there is an increased in cytokines released contributing to retinal degeneration (Ibrahim *et al.*, 2011; Tang and Kern, 2011). Having this into account, we intended to study the anti-inflammatory effect of Pyr-sulf using an *in vitro* approach, with D407 RPE cells, by evaluating the relative expression of IL-1β and IL-8, pro-inflammatory cytokines.

Figure 4.6 represents the levels of gene expression of IL-1β with and without treatment with Pyr-sulf.

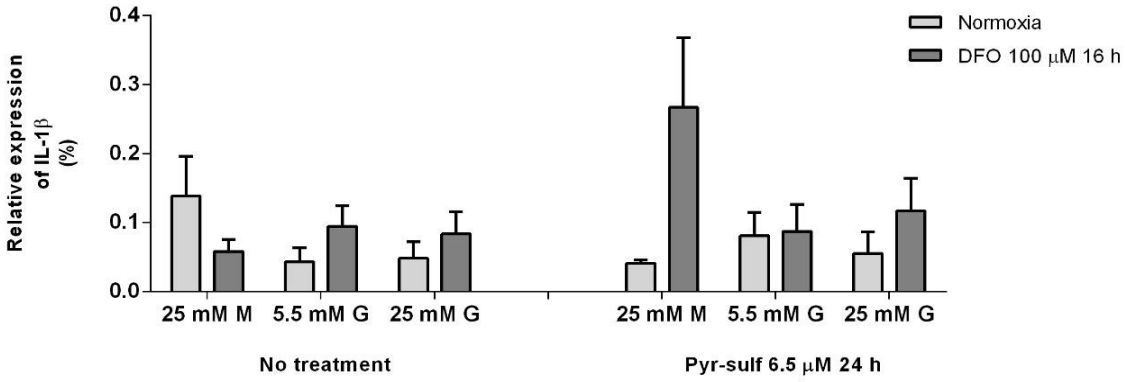

Figure 4.6 Pyr-sulf treatment does not alter gene expression of IL-1β. RT-qPCR analysis of IL-1β mRNA expression in D407 cells after Pyr-sulf treatment (6.5 μM 24 h). β-Actin was used as housekeeping control. N=4. M, Mannitol (osmolarity control) and G, Glucose.

Knowing that IL-1β is released in diabetic environment (Vincent and Mohr, 2007), we performed a RT-qPCR to evaluate the effect of Pyr-sulf in the expression of this cytokine. Figure 4.6 shows that in cells without treatment, there is a slight increase in the mRNA expression of IL-1β in hypoxia conditions, suggesting the activation of the inflammatory state in these conditions. However, in untreated cells cultured in high glucose (25 mM G) under hypoxia there is no significant difference in IL-1β mRNA expression when compared with cells cultured in low

glucose (5.5 mM G) under hypoxia, which is opposite what we are expecting. We were expecting the levels of IL-1β to be higher in the pathological condition because chronic exposure to large amounts of glucose leads to a chronic inflammatory state. According to the literature, the peak of inflammation in D407 cells exposed to 25 mM glucose is at 4 hours after the induction of hyperglycemia (Busik, Mohr and Grant, 2008). We performed the RT-qPCR 24 h after inducing hyperglycemia which may suggest that the peak of inflammation had already passed, and the cells developed a mechanism of habituation to the glucose concentration in the medium.

After treatment with Pyr-sulf, in cells cultured in low glucose (5.5 mM G) there is no difference in mRNA expression of IL-1β when compared with the same untreated condition or treated condition under normoxia. However, the treatment increases the expression of IL-1β in high glucose (25 mM G) under hypoxia, when compared with treated cells in high glucose (25 mM) under normoxia, however when compared with the correspondent untreated cells there is no difference. These results suggest that Pyr-sulf have no influence in mRNA expression of IL-1β and treatment is not working.

Figure 4.7 represents the levels of gene expression of IL-8 with and without treatment with Pyr-sulf.

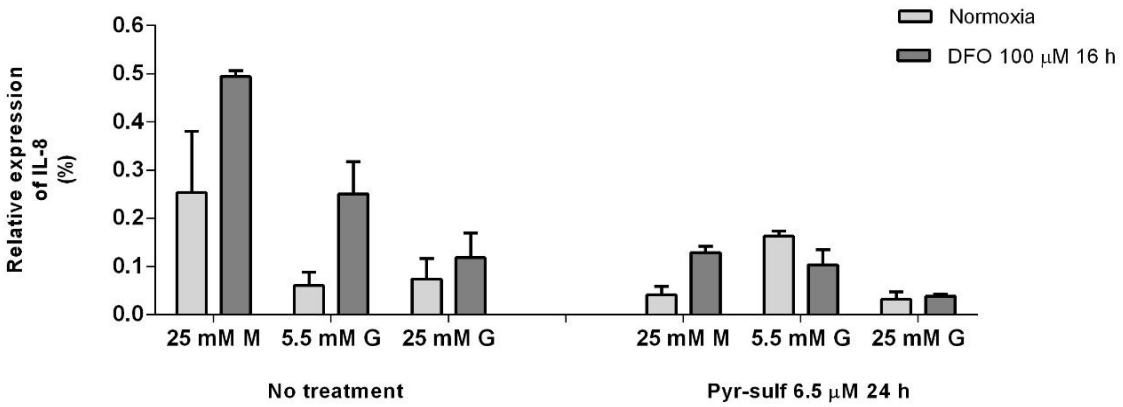

Figure 4.7 Pyr-sulf treatment reduces inflammation in hypoxia condition. RT-qPCR analysis of IL-8 mRNA expression in D407 cells after Pyr-sulf treatment (6.5 μM 24 h). β-Actin was used as housekeeping control. N=4. M, Mannitol (osmolarity control) and G, Glucose.

Also IL-8 is released in diabetic environment (Vincent and Mohr, 2007), so we performed a RT-qPCR to evaluate the effect of Pyr-sulf in the expression of this cytokine. Figure 4.7 shows that in cells without treatment the mRNA expression of IL-8 increases in hypoxia conditions. When untreated cells are in low glucose (5.5 mM G) under hypoxia there is an increase in IL-8 relative expression when compared with normoxia, suggesting the activation of the inflammatory state in hypoxia conditions. However, in untreated cells cultured in high glucose (25 mM G) under hypoxia there is a decrease of IL-8 mRNA expression when compared with cells cultured in low glucose (5.5 mM G) under hypoxia, which is opposite what we are

expecting and is according with what happen in the IL-1β mRNA expression in the same conditions, suggesting that the peak of inflammation had already passed.

After treatment with Pyr-sulf, the mRNA expression of IL-8 decrease in hypoxia conditions. For cells in low glucose (5.5 mM G) under hypoxia, the IL-8 relative expression is lower when compared with cells without treatment in low glucose (5.5 mM G) under hypoxia. In treated cells under high glucose (25 mM G) and hypoxia, the IL-8 expression is also lower when compared with cells without treatment under high glucose (25 mM G) and hypoxia. These results suggest that treatment with Pyr-sulf reduces the expression of IL-8 in hypoxia conditions and consequently the inflammation.

As described in literature (Muto *et al.*, 2015), the expression of IL-8 can be induced by IL-1β. Our results show no differences in IL-1β mRNA expression after the onset of hyperglycemia and hypoxia. After the treatment, the expression of IL-1β increases in Mannitol under hypoxia, which is not expected, suggesting some problems with the protocol and consequently no effect of Pyr-sulf in this situation However there are differences in IL-8 expression. These results suggest a different regulation level of IL-8 where Pyr-sulf has effect.

4.5. Treatment with Pyr-sulf decreases inflammation in $Ins2^{Akita}$ 8 months-old mice

Similarly, to the *in vitro* experiments, we intended to evaluate the effects of the treatment with Pyr-sulf in the protein levels of our target molecules in an *in vivo* model of DR. For that purpose we used $Ins2^{Akita}$ diabetic mice model and/or non-diabetic (WT) mice with 8 and 9 months-old, when DR features are established (Barber *et al.*, 2005). In both timepoints we have analyzed the expression of pro- and anti-angiogenic proteins, VEGF and PEDF respectively, the expression of the glucose transporter, GLUT1 and the expression of an inflammatory marker, Iba1 by Western blot. The retina extraction was performed two weeks after the Pyr-sulf subretinal injection as described in section 3.5.4.

Figure 4.8 represents the levels of protein expression of PEDF and VEGF (A), GLUT1 (B) and Iba1 (C) two weeks after the Pyr-sulf subretinal injection.

Because we were not able to perform the experiments in age-matched non-diabetic mice, we will only describe our observations regarding Ins2Akita mice, comparing the non-injected eye with the treated eye. Treatment with Pyr-sulf does not affect the expression of PEDF and VEGF proteins in diabetic mice (Figure 4.8 A) when compared with non-injected conditions. The PEDF results are consistent with those obtained with PEDF expression in D407 RPE cells (Figure 4.3 B), contrary to what happens with VEGF expression *in vitro* (Figure 4.4 B). In the same manner, the treatment with Pyr-sulf does not seem to affect GLUT1 protein expression (Figure 4.8 B).

These results need further validation due to the low number of experiment animals. Also, at this age, the retina of these animals is at an advanced stage of DR, affecting the expression of our proteins of interest (PEDF, VEGF and GLUT1).

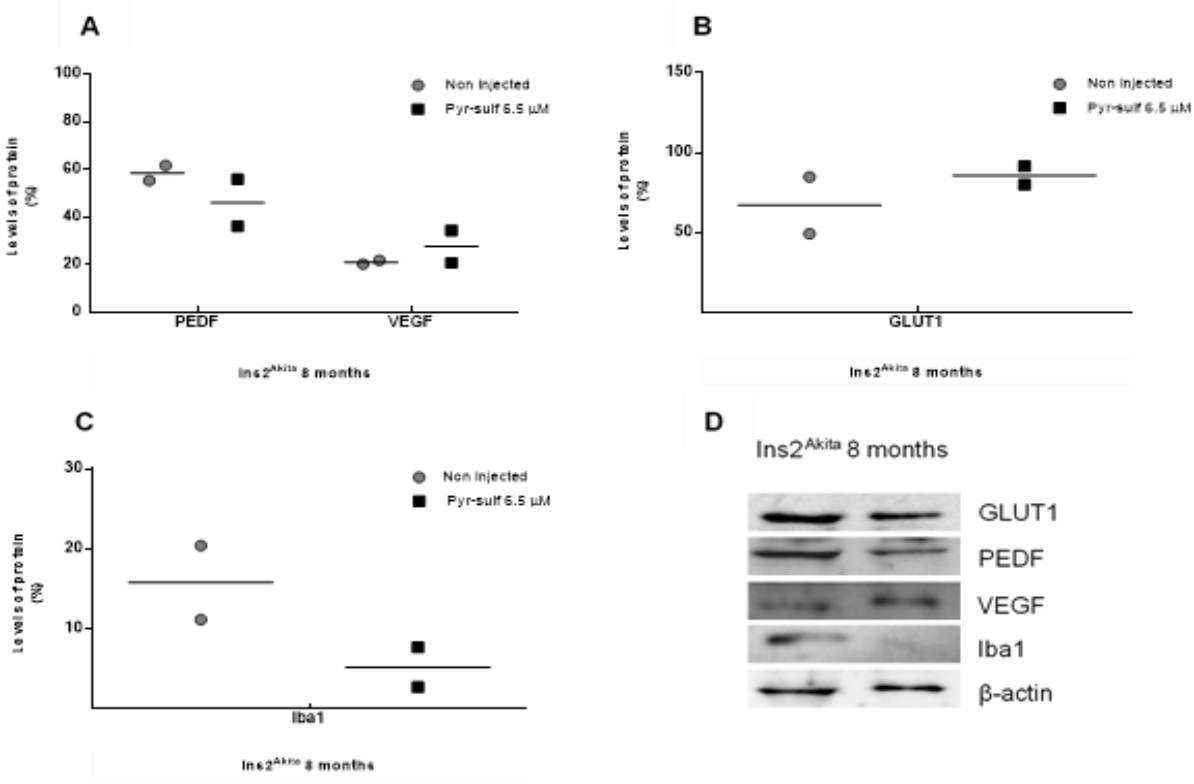

Figure 4.8 PEDF, VEGF, GLUT1 and Iba1 expression in 8 months Ins2^{Akita}, 2 weeks after Pyr-sulf injection. (A) Represents the quantitative data normalized to the intensity of β-Actin of PEDF and VEGF, for Ins2^{Akita} mouse with 8 months. N=2. (B) Represents GLUT1 quantitative data normalized to the intensity of β-Actin of GLUT1, for Ins2^{Akita} mouse with 8 months. N=2. (C) Represents Iba1 quantitative data normalized to the intensity of β-Actin of Iba1, for Ins2^{Akita} mouse with 8 months. N=2. (D) Representative Western blot images of PEDF, VEGF, GLUT1 and Iba1 for non-injected and injected conditions (Pyr-sulf, 6.5 µM).

Despite the results obtained for other proteins, Figure 4.8 C shows a decrease in the levels of Iba1 after treatment with Pyr-sulf, although not statiscally significant, probably due to the low number of experimental animals. Iba1 is a pro-inflammatory protein released when the microglia is activated. Being a marker for inflammation, the decrease in its protein levels after Pyr-sulf injection highlights the anti-inflammatory efficacy of this phenolic metabolite, already indicated by the *in vitro* studies for IL-8, another inflammation marker (Figure 4.7). As mentioned at the beginning of this section, these results need the support of the analysis of age-match non-diabetic mice in the same conditions. Otherwise we cannot draw any conclusions.

4.6. Treatment with Pyr-sulf has no effect in Ins2^{Akita} and in age-matched wild-type 9 months-old mice

To confirm our *in vitro* observations, we performed some preliminary *in vivo* studies with diabetic mice models Ins2^{Akita} and with age-matched wild-type mice 9 months-old. We have analyzed PEDF, VEGF, GLUT1 and Iba1 proteins expression by Western blot.

Figure 4.9 represents the levels of protein expression of PEDF and VEGF (A), GLUT1 (B) and Iba1 (C) two weeks after the Pyr-sulf subretinal injection.

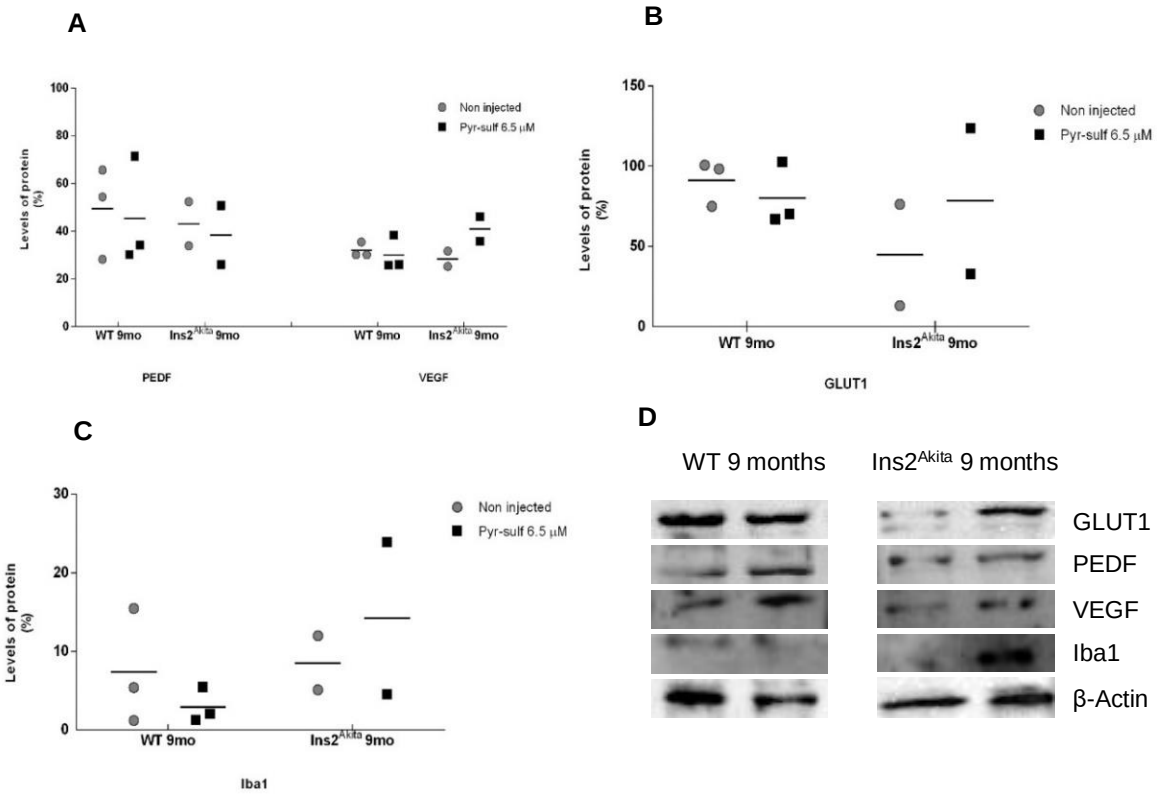

Figure 4.9 PEDF, VEGF, GLUT1 and Iba1 expression in 9 months Ins2^{Akita} and wild type mouse retina, 2 weeks after Pyr-sulf injection. (A) Represents PEDF and VEGF quantitative data normalized to the intensity of β-Actin of PEDF and VEGF, for wild-type and Ins2^{Akita} mouse with 9 months. N=2 to 3. (B) Represents GLUT1 quantitative data normalized to the intensity of β-Actin of GLUT1, for wild-type and Ins2^{Akita} mouse with 9 months. N=2 to 3. (C) Represents Iba1 quantitative data normalized to the intensity of β-Actin of Iba1, for wild-type and Ins2^{Akita} mouse with 9 months. N=2 to 3. (D) Representative Western blot images of PEDF, VEGF, GLUT1 and Iba1 of non-injected and injected conditions, for wild-type and Ins2^{Akita} mice.

Figure 4.9 A, shows no differences in PEDF and VEGF protein expression when treated eyes of $Ins2^{Akita}$ mice are compared with the non-injected eye and age-match wild-type animals. The results with PEDF are according to those obtain for PEDF protein expression *in vitro* (Figure 4.3 B) and with those obtain for $Ins2^{Akita}$ 8 months mice (Figure 4.8 A). Also, the VEGF expression in $Ins2^{Akita}$ mice at this timepoint is similar to what we observe in 8-months old mice (Figure 4.8 A). The results *in vivo* after the treatment are according with those obtained *in vitro* (Figure 4.3 B and Figure 4.4 B). The treatment with Pyr-sulf does not seem to increase PEDF expression, but we could further increase the number of animals tested to corroborate the results. In this way, we could later explore the effect of the treatment on VEGF expression *in vivo*, increasing the number of injections, or inject Pyr-sulf at different timepoints. In this study we are analyzing the expression of PEDF and VEGF in the retina, however these proteins are secreted by RPE. Additionally, we can analyze its expression in the mice RPE cells to elucidate the results obtained. In fact, other studies in our lab show differences in VEGF and PEDF expression in the retina and in the RPE. For example, in the retina of 12 months mice the results shown an increase in VEGF levels, however in the RPE this increase was at 9- and 12-months mice (data not shown).

Treatment with Pyr-sulf increases GLUT1 expression in diabetic mice when compared with non-injected, however there is no difference when compared with age-matched wild-type mice. These results show that Pyr-sulf injection does not affect GLUT1 protein expression (Figure 4.9 B). Although these results are supported by the literature (Calado *et al.*, 2016), they are in according with the results obtained with D407 RPE cells for GLUT1 expression (Figure 4.5) and we can conclude that Pyr-sulf treatment is not effective in decreasing GLUT1 protein expression.

In Figure 4.9 C the expression of Iba1 decrease in wild-type mice after Pyr-sulf treatment which is according with Pyr-sulf anti-inflammatory properties. However, in diabetic mice the expression of Iba1 tends to increase, which does not corroborate what we have observed for 8-month-old animals. This may be related to the low number of animals analyzed and the natural variability among animals. Beside this, the injection is an invasive procedure that causes a natural inflammatory response in the animals, which certainly influences the results obtained. To further corroborate this, we can establish a control (sham-injected animal) for the subretinal injection. In this case we perform the injection with PBS 1x instead of the phenolic metabolite and normalize the results with this condition to eliminate probable interferences of the natural inflammation.

5. General discussion

Diabetic Retinopathy is a blood-retinal barrier disorder that affects 90% of type 1 diabetes patients, and it is the leading cause of blindness worldwide (Cheung, Mitchell and Wong, 2010; Garg and Davis, 2009). This chronic and progressive disease is mostly characterized by ischemia, microaneurysms, hemorrhages, neovascularization, increased vascular permeability and, as for all chronic diseases, inflammation. It is well known that hyperglycemia and hypoxia are the main keys for the development of DR, however the pathophysiological mechanism behind this disorder remains unclear (Cheung, Mitchell and Wong, 2010). In DR, the ischemia leads to oxygen privation and to satisfy tissue metabolic requirements signaling pathways are activated to induce new blood vessels formation in the retina. During this process, pro-angiogenic factor, VEGF, is overexpressed while the anti-angiogenic protein, PEDF, is downregulated. This imbalance between pro- and anti-angiogenic proteins is responsible for neovascularization in the eye and consequently vision loss (Farjo and Ma, 2010). In addition to VEGF and PEDF, the only glucose transporter (GLUT1) in RPE cells is also affected in DR (Ban and Rizzolo, 2000; Sone, Deo and Kumagai, 2018).

Among the last decades, polyphenols have been widely studied due to its incredible properties. Has been identified more than 8000 polyphenolic compounds in plants. It is known that berries are a great source of polyphenols which makes them a good alternative for healthy diet and to prevent the progression of several diseases (Manach *et al.*, 2004; Rio, Del *et al.*, 2013). From a study with berry fruits, some researchers shown that Cat-sulf and Pyr-Sulf are the most abundant phenolic metabolites in the urine and plasma of some volunteers (Pimpão *et al.*, 2014, 2015). Thus, based on these previously results we have tested the effect of these metabolites in DR hallmarks, like angiogenesis and inflammation.

To understand the effect of polyphenols in DR conditions, we perform *in vitro* studies using D407 cells, a spontaneously transformed RPE cell line derived from a primary culture of human RPE cells (Davis *et al.*, 1995). D407 cells were exposed to different concentrations of glucose, 5.5 mM D-Glucose or 25 mM D-Glucose to mimic physiological or pathological diabetic conditions, respectively. Furthermore, the cells were also exposed to DFO 100 µM to induce hypoxia and simulate retinal ischemia, characteristic of DR. Also, we tested the effect of phenolic metabolites in a mice model of advanced DR, using 8- and 9-month old $Ins2^{Akita}$ mice, by subretinal injection.

Before studying the effects of polyphenol treatment on RPE cells we wanted to test whether these compounds had influence on the metabolic activity of D407 cells and optimize the time of hypoxia that does not induce cell death.

Use of the MTT tetrazolium compound to measure the number of viable cells in culture was first described by Mosmann in 1983 (Mosmann, 1983). We perform this assay to evaluate the effect of the phenolic metabolites in D407 cell viability. The treatment with Cat-sulf show significantly differences in cell viability, 24 h after the treatment (Figure 4.1), suggesting that Cat-sulf affects the metabolic activity of D407 cells.

Despite the results obtain with Cat-sulf, the treatment with Pyr-sulf does not influence the D407 cell viability (Figure 4.2). These results may suggest that Pyr-sulf is metabolized differently by cells, perhaps because of their different chemical structures. Based on previous studies we conclude that the metabolization of Pyr is more time consuming than that of the Cat-sulf, because Pyr-sulf has two different ways of metabolization, in one of them it is metabolized in Cat-sulf (Figueira *et al.*, 2017). We hypothesized that an increase in the time of exposure of the treatment to 48 h will show a different outcome. In fact, previous studies in our laboratory in which we incubated Cat-sulf for 48 h (data not shown) allowed us to conclude that cell viability is significantly affected.

MTT assay was also performed to assess the time of cell exposure to hypoxia. We tested two different hypoxia timepoints, and our results show that after 24 h of hypoxia the cell viability is lower than with 16 h of hypoxia (Figure 4.1and Figure 4.2 B). In order to verify if hypoxia is being correctly induced with DFO, we could evaluate the expression of Hypoxia-Inducible Factor 1-alpha (HIF1-α). This factor is encoded by the HIF1-α gene and plays an important role in response to systemic oxygen levels. HIF1-α is responsible for the transcription of several genes including the important VEGF, to induce angiogenesis in oxygen privation situations - so if VEGF is overexpressed, HIF1-α will also be (Kallio *et al.*, 1997; Lee *et al.*, 2006). In addition to allowing us to confirm hypoxia induction, analyzing the expression of HIF1-α will also allow us to normalize our results against these conditions, to discard the influence of hypoxia on the expression of the proteins under study.

Taking the previous results into account we decided to focus our attention and further experiments only in one phenolic metabolite, Pyr-sulf, and to induce hypoxia for 16 hours to be sure that the metabolic activity of the cells is not compromised.

After cell culture in the conditions previously described, we have performed the quantitative analysis of protein and mRNA levels of the gene of interest by Western blot and RT-qPCR, respectively. We analyzed the mRNA expression and protein levels to evaluate the effect of Pyr-sulf in restoring the balance between VEGF and PEDF. Our results for mRNA expression of PEDF (Figure 4.3 A), although not statistically significant, showed an increase in mRNA relative expression of PEDF when treated cells are in diabetic conditions, compared with the high glucose condition in normoxia treated cells. These results showed a promising beneficial effect of treatment with Pyr-sulf in re-establishing the normal regulation of this anti-angiogenic factor. But when assessing the levels of PEDF at a post-translational level (Figure 4.3 B) we only see a correlation with mRNA results in cells without treatment under hypoxia, where the levels of protein are significantly lower in high glucose than in low glucose. These results confirm once more that PEDF is downregulated in high glucose and hypoxia situations. Despite this, and unlike what happens with the mRNA levels, the treatment with Pyr-sulf in general decreases PEDF expression. Further studies will be needed to confirm the effect of Pyr-sulf treatment on PEDF expression, namely studies on the secretory function of RPE, since PEDF is one of the most important secreted proteins of this retinal cell layer.

It was previously shown that VEGF levels are increased in diabetic conditions (Cheung, Mitchell and Wong, 2010). Our results show that hypoxia induces an increase in VEGF mRNA in cells without treatment, like we were expecting (Figure 4.4 A). However, there is a decrease in VEGF mRNA in cells in high glucose condition under hypoxia, compared with cells in low glucose under hypoxia. In high glucose medium we were expecting to have a stronger increase in VEGF mRNA expression, because this factor controls the glucose passage to the retina. After the treatment with Pyr-sulf there was no differences in VEGF mRNA expression, suggesting that Pyr-sulf has no influence at mRNA level.

When we analyze VEGF protein expression (Figure 4.4 B) there is a slight increase in VEGF levels in low glucose when comparing hypoxia with normoxia in cells without treatment, which is in accordance with the RT-qPCR results. Despite the decrease at mRNA levels, at protein level there is a slight increase in VEGF protein in the cells cultured in high glucose compared with the cells cultured in low glucose medium under hypoxia. This may suggest that high glucose affects VEGF expression at protein level. After Pyr-sulf treatment, there is significant differences in protein expression. The VEGF expression not only decreases in diabetic conditions when compared with same condition without treatment, but also decreases in cells under normoxia with high glucose concentration. Contrary to what happen with PEDF, Pyr-sulf seems to influence VEGF expression at pos-translational levels, with a potential effect on the molecular level of neovascularization.

To evaluate the effect of Pyr-sulf in expression of GLUT1 we performed a Western blot analysis (Figure 4.5). Our results shown an increase in GLUT1 protein in high glucose under hypoxia, in cells without treatment. This result is in accordance with previously work in our lab, where it was shown that hypoxia induces an increase in GLUT1 protein levels in high glucose medium. This suggest that, diabetic environment contributes to increase GLUT1 protein levels due to the high concentration of glucose in the medium and oxygen privation. After treatment with Pyr-sulf, GLUT1 levels decreases in all conditions compared to untreated cells, suggesting that there is a tendency, although not statiscally significant, for treatment with Pyr-sulf decrease the expression of GLUT1 protein, highlighting the beneficial effect of Pyr-sulf in controlling the transport of glucose in DR. One easy experiment we could do to confirm these results is to assess glucose consumption of RPE cells and compare treated and no treated cells with the phenolic metabolite.

Inflammation is a hallmark of DR, since hyperglycemia and hypoxia lead to microglia activation and release of pro-inflammatory molecules. IL-1β and IL-8 are both cytokines secreted cells in a chronic inflammatory state, functioning as an inflammation marker. Knowing that IL-1β and IL-8 are released in diabetic environment (Vincent and Mohr, 2007), we have performed a RT-qPCR to evaluate the effect of Pyr-sulf in the expression of these cytokines. Our results showed no differences in IL-1β mRNA levels in cells without treatment under hypoxia but also showed an increase in IL-8 mRNA expression in the same conditions. However, in untreated cells in high glucose under hypoxia the expression of these cytokines is not higher when compared with low glucose under hypoxia. We expected the levels of IL-1β

and IL-8 to be higher in high glucose (Figure 4.6 and Figure 4.7) because chronic exposure to large amounts of glucose leads to a chronic inflammatory state. According to the literature, the peak of inflammation in D407 cells exposed to 25 mM glucose is at 4 hours after the induction of hyperglycemia (Busik, Mohr and Grant, 2008). We performed the RT-qPCR 24 h after inducing hyperglycemia which may suggest that the peak of inflammation had already passed, and the cells developed a mechanism of habituation to the glucose concentration in the medium.

After treatment with Pyr-sulf, although we did not see differences in IL-1β (Figure 4.6) expression, IL-8 mRNA expression decreases in cells under hypoxia conditions compared with cells without treatment in low glucose under hypoxia (Figure 4.7). This result shows the capacity of Pyr-sulf in reducing inflammatory states in hypoxia.

The results obtained for the expression of IL-1β (Figure 4.6) are not according with what we are expecting, which may indicate that inflammation is not being induced properly. To corroborate the results, we can stimulate cells with LPS (lipopolyssacharide), the principle component of the membrane of gram-negative bacteria's that induces a response by the immune system, and then evaluate the expression of inflammatory markers and the effect of Pyr-sulf in reduce inflammation.

To confirm our *in vitro* observations, we have performed some preliminary *in vivo* studies with diabetic mice models $Ins2^{Akita}$ and with age-matched wild-type mice, at 8 and 9 months-old.

Treatment with Pyr-sulf does not affect the expression of PEDF and VEGF proteins in diabetic mice (Figure 4.8 and Figure 4.9 A) when compared with non-injected condition. The PEDF results are consistent with those obtained with PEDF expression in D407 RPE cells (Figure 4.3 B), contrary to what happens with VEGF expression *in vitro* (Figure 4.4 B). Our results for the expression of GLUT1 protein seems to show no differences in GLUT1 expression after the injection with Pyr-sulf in both 8 and 9 months $Ins2^{Akita}$, showing that the treatment does not affect GLUT1 expression, which is also associated with the severe state of the disease installed in these ages. At this age, the animal's retina is aged, which can explain the results obtained for PEDF, VEGF and GLUT1 expression in the retina of $Ins2^{Akita}$ diabetic mice (Kakoki *et al.*, 2006). Moreover, the results we have obtained for the expression of GLUT1 corroborates previous studies from our lab for RPE cells and $Ins2^{Akita}$ mice (Calado *et al.*, 2016).

However, in 8 months $Ins2^{Akita}$ mice the treatment with Pyr-sulf decreases Iba1 protein levels (Figure 4.8 C). Iba1 is a pro-inflammatory protein released when the microglia is activated, being a marker for inflammation. After treatment, there is a reduction in Iba1 levels suggesting the anti-inflammatory efficacy of Pyr-sulf. In 9-month mice this reduction is only observed in wild-type aged-matched (Figure 4.9 C). These results together with our *in vitro* findings, and with work of others (Pandey e Rizvi, 2014) confirm the anti-inflammatory effect of polyphenols, such as Pyr-sulf. Since inflammation is a hallmark in DR, Pyr-sulf can have beneficial effects in preventing this disease.

Overall, the results observed in the *in vivo* model may be explained by the low number of animals analyzed so far. However, since inflammation arises in early stages of RD it would be

important to study the expression of the proteins involved in the pathophysiology of the disease in animals aged less than 8 months.

Since inflammation is a hallmark of all chronic diseases and our results confirm the great anti-inflammatory potential of Pyr-sulf, we can explore more this feature. In this work we only analyzed two different inflammatory biomarkers, however there are more to be explored. In the future, we can analyze the effect of Pyr-sulf in the expression of other inflammation markers known being increased in DR such as tumor necrosis factor alpha (TNF-α). TNF-α is an important cytokine produced by activated macrophages, neurons and other cell types (Demircan *et al.*, 2006). This protein is activated even in low grade chronic inflammation, its first function is to regulate the immune cells and induce inflammation. It is proved that in hyperglycemia TNF-α induce insulin resistance (Feinstein *et al.*, 1993), so study the effects of anti-inflammatory compounds, such as Pyr-sulf is imperative to improve complications associated with diabetes. Also interleukin 6 is known to be involved in chronic inflammation processes in disease (Tang and Kern, 2011). Interleukin 6 is a pleiotropic protein released by macrophages and T-cells in the local of the injury together with IL-1β and TNF-α (Tanaka, Narazaki and Kishimoto, 2014). To confirm the anti-inflammatory effect of Pyr-sulf treatment we can also evaluate the expression of anti-inflammatory biomarkers, such as interleukyne-10, which in treated conditions may be increased. Interleukin 10 downregulate the expression of pro-inflammatory cytokines such as IL-1β (Yan and Mao, 2014). Another important pathway is the NF-kβ signaling pathway. This pathway is considered a proinflammatory signaling pathway by activation of several proinflammatory molecules, such as, IL-8 (Lawrence, 2009; Muto *et al.*, 2015), so it could be a therapeutic target to reduce inflammation by Pyr-sulf treatment.

Furthermore, Glial Fibrillary Acidic Protein (GFAP) produced by astrocytes is responsible for the regeneration of glial cells after injury, being essential in cell support (Middeldorp and Hol, 2011). However, in chronic situations its production can be deregulated, so studying the effect of Pyr-sulf in GFAP expression can elucidate the great potential of this metabolite. Previous work in our lab, shown a decrease in GFAP expression in diabetic mice with 6 months-old. In the future, we can test the effect of Pyr-sulf in increasing GFAP expression and consequently ameliorate the astrocytes function to support cells in injury.

The accumulation of ROS, by disturbance in the normal redox state of cells and the failure of the antioxidant defense system are the main characteristics of oxidative stress, also an important hallmark of DR. The production of ROS triggers pro-inflammatory responses that may be involved in chronic diseases like diabetes, and development of therapies that modulate their production can be an interesting approach (Obrenovich *et al.*, 2011; Rodrigo, Miranda and Vergara, 2011). In normal conditions, endogenous antioxidants like enzymes are responsible for reducing these species, however polyphenols can be an exogenous source of anti-oxidants and act against oxidative stress (Macedo *et al.*, 2015; Obrenovich *et al.*, 2011; Rodrigo, Miranda e Vergara, 2011). In future works we are planning to evaluate the effect of Pyr-sulf in oxidative stress. There is several techniques that allows to analyze the formation of this species. We can use for example specific antibodies to evaluate the changes in specific proteins caused by

ROS, or specific tag and then immunodetection, among others. However, the experiences have to be clearly planned because redox signaling is an essential part of normal homeostasis, so any therapeutic design must be constructed to distinguish pathological redox from normal. Based on these facts, and since oxidative stress is a hallmark of DR, study the effect of Pyr-sulf in decreasing ROS may give an important advantage in developing therapies to ameliorate the outcomes of DR.

6. Conclusion

This work allows to confirm the decrease in PEDF levels in RPE cells under diabetic conditions and, although not statiscally significant, we observed an increased in PEDF mRNA expression after the treatment with Pyr-sulf. However, treatment with Pyr-sulf has no effect in PEDF protein expression, suggesting that Pyr-sulf only affects PEDF at transcriptional levels. We also confirmed that high glucose increases VEGF levels at protein manner, as we were expecting. Unlike to what happens with PEDF, treatment with Pyr-sulf influences VEGF protein expression, suggesting that Pyr-sulf affects VEGF at pos-translational levels. Regarding glucose intake, although not statistically significant, treatment with Pyr-sulf appears to decrease GLUT1 levels, evidencing the beneficial potential of this polyphenol for controlling the deleterious glucose intake levels. Despite results with D407 cells, *in vivo* treatment with Pyr-sulf appears to have no influence on studied proteins, which can be associated with the advanced state of the disease and because at this stage inflammation is no longer the main cause of physiological changes.

However, the great anti-inflammatory potential of Pyr-sulf is shown both in *in vivo* and *in vitro*. The IL-8 mRNA expression decreases under hypoxia conditions in RPE cells and in diabetic mice, Iba1 protein expression also decreases after the treatment, suggesting the beneficial effect of Pyr-sulf in reducing inflammation.

Further studies will focus on the effect of Pyr-sulf on ROS and on another inflammation markers as well as testing another incubating time of polyphenols in cells.

Altogether, our results show the capacity of Pyr-sulf in ameliorating the effects of DR, especially the anti-inflammatory potential, adding to the potential of this compound in preventing the development of DR.

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